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

JUL 15 1999

MEMORANDUM

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

SUBJECT: Review of Additional Data from AgrEvo USA on the Cry9C Protein Expressed in Corn

TO: Mike Mendelsohn
Regulatory Action Leader
Biopesticides & Pollution Prevention Division
(7511C)

FROM: John L. Kough, Ph.D., Biologist
Microbial Pesticide Branch
Biopesticides & Pollution Prevention Division
(7511C)

and

Michael T. Watson, Ph.D., Plant Pathologist
Microbial Pesticide Branch
Biopesticides & Pollution Prevention Division
(7511C)

DATA REVIEW RECORD

Active Ingredients:	Cry9C protein and the genetic material necessary for its production
Product Name:	StarLink™ Corn
Company Name:	AgrEvo USA Company, Wilmington, DE
ID No:	9F05050
Submission No:	S554804
Chemical No:	006466
DP Barcode:	D252309

THIS REVIEW CONTAINS CONFIDENTIAL BUSINESS INFORMATION
(CBI that was present in an attached DER has been removed.)

ACTION REQUESTED

To review the information submitted by AgrEvo for allergenicity and subchronic toxicity related issues of the Cry9C protein as expressed in corn.

BACKGROUND

AgrEvo has been given a registration for use of the Cry9C gene and expressed protein in Starlink™ corn. The accompanying food tolerance exemption was for use of the protein only in feed corn as

issues related to potential allergenicity of direct human consumption were as yet unresolved. In the interim the company provided summary information of results from a study conducted in the Netherlands which indicated the Cry9C protein was identified as a food allergen in the brown Norway rat model and also appeared in the blood stream of animals dosed orally with Cry9C. This submission includes the full report of these studies.

BPPD RECOMMENDATIONS

The data presented do not add substantially to that presented in the previous review of Cry 9C for confirming or refuting the potential of Cry9C to be a food allergen. The indications are confirmed that Cry9C is not substantially altered by incubation in gastric or intestinal fluids. The gastric incubation did degrade about 15 to 25% of the protein as measured by scanning densitometry compared to the controls. In dosed animals the 68kDa form of Cry9C is converted to a 55kDa form perhaps by the microbial action of the intestines but no further degradation is evident. No significant uptake into the blood of any immunoreactive form of Cry9C is indicated by the bioavailability study. The 30-day dietary study presented no adverse effects except a change in the fat pads of animals in the high dose groups and an altered level of ketone in the blood which may be indicative of altered carbohydrate metabolism. The brown Norway rat model is not a validated test for food allergy at this point and the study submitted is significantly flawed by contamination of the corn tissue controls, poor description of the experimental protocols and possibly immunization procedures themselves. In addition, the protein expression data are questionable due to apparent contamination of the controls. It is recommended that the company proceed with developing a monitoring program to detect if any food allergy occurs from human exposure to their pesticidal trait. In addition, the company should continue to follow developments in the food allergenicity field and be aware that more definitive tests may be required in the future.

SUMMARY OF REVIEWS

MRID 447140-01: The summary information contained 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 not 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 of known allergens. Except for the reviews by BIBRA International, Dr. Andrew Cockburn, and Dr. Samuel B. Lehrer, all of the other information has been submitted previously, or under different MRID numbers in this submission. Several of the studies enclosed in this submission also require supplementary information or explanation.

As with much of the data and information submitted in previously and with this package, it is not possible to reach a definite conclusion regarding the allergenic potential of the Cry9C protein based upon the supplementary information. As can be seen from the enclosed letter and reviews, even expert analysis of potential allergenicity 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

2 of 6

the available information. Further data and clarification must be provided to aide in such a determination.
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.

MRID 447140-02: The results of intraperitoneal injection of corn powder extracts into BN rats indicate that both the control and transgenic corn powders are able to induce IgE or reagininc antibody responses by the PCA assay. The use of corn powder immunogen decreases the rate of the immune response to the Cry9C protein compared to the bacterial preparation. However, the lowest responding dose for Cry9C was similar for the two preparations (between 0.1 and 0.4 µg Cry9C). The control challenge test with the heterologous antigen of control corn powder or transgenic corn powder in the day 42 sera samples indicate that there was significant reactivity from the corn portion of the extracts themselves in the PCA assay. It is unclear, given this background reactivity, how suppositions can be made about the reactivity of the Cry9C protein alone. The PCA results from oral sensitization with ovalbumin II, control corn extract, bacterial Cry9C and transgenic corn (apparently supplemented with bacterial Cry9C) indicated that an IgE or reagin antibody response was elicited in naïve Sprague-Dawley rats. Ovalbumin sensitized serum produced a low frequency of responders and a weak dose response between the 5.0 and 50.0 mg/kg dose levels on days 28 through 42. The control corn also produced a positive oral sensization response but this was only examined at the 50 mg/kg dose. Oral dosing with bacterial Cry9C gave a positive PCA response as did the Cry9C amended transgenic corn extract. The frequency of response to bacterial Cry9C began to diminish in day 42 sera. The Cry9C amended transgenic corn had a higher frequency of responders and the frequency remained high on day 42 PCA response. Western blot analysis indicated that Cry9C protein bands could be recognized in the rat sera from both exposure routes.

CLASSIFICATION: Supplemental. A more detailed description of the materials and methods, especially how the PCA system was utilized in these experiments, is needed. Specifically: 1) how long after the serum injections were the challenge antigen injections done; 2) what was the concentration of Cry9C in the bacterial and corn plant powder preparations; 3) The source of the test animals and their care during the test were not described; 4) How many naïve Sprague Dawley rats were used for each serum tested; 5) What were the individual animal responses; 6) The original gel for the western blot assay should be provided.

MRID 447140-03: The testimonial letters submitted by employees of the Garst Seed Company indicate that 1980 people with considerable direct exposure to corn seed and plant parts including tassels and pollen have not experienced adverse or allergic responses they could directly attribute to exposure to the Cry9C protein in StarLink™ corn.

CLASSIFICATION: Acceptable. The company is reminded that they are still responsible for reporting any incidents of hypersensitivity or other adverse effects they are aware has resulted from exposure to the Cry9C protein.

MRID 4473430-1: The conclusions drawn by AgrEvo in this study indicate that the addition of unlabeled Cry9C in the displacement of the labeled (biotinylated) Cry9C, demonstrating that the

Cry9C binds specifically and saturably to *Ostrinia nubilalus* BBMVs. In addition, the addition of unlabeled Cry9C did not result in the displacement of the labeled Cry9C, demonstrating that Cry9C shows only a small amount of non-specific binding, and does not bind specifically and saturably to mouse intestinal BBMVs.

However, the copy of the electrophoretogram provided is not of very good quality, and therefore, it is impossible for BPPD to reach these same conclusions. Assuming that clear figures are available, it is also unclear to BPPD what AgrEvo means by "displacement" by the unlabeled Cry9C in the insect BBMV samples. The term "displacement" indicates an active process where there is a mechanism responsible for dislodging labeled proteins and allowing these displaced proteins to be replaced by the unlabeled Cry9C proteins, or some similar process. There is no discussion in this submission which addresses the displacement issue. Further explanation/discussion should be provided.

CLASSIFICATION: SUPPLEMENTAL. This submission can be upgraded to ACCEPTABLE with submission of adequate figures and additional information to support the conclusion reached regarding the binding potential of Cry9C to the tissues tested. Supplemental data/information should include:

1. Adequate figures (originals or high quality reproductions) of the chemiluminescent results that will allow for clear differentiation between protein signals and background "noise".
2. Molecular weight markers to clearly identify the molecular weights of the identified protein bands. Also, identification of any "control" protein samples used for comparison to test samples.
3. Explanation/identification of the additional bands present in the Mouse BBMV samples.
4. Further discussion and justification regarding the "displacement" of the labeled Cry9C proteins by the unlabeled proteins.

MRID 447343-02:
An acute intravenous toxicity study was conducted with proteins derived from two *B. thuringiensis* species and from BSA. From the data presented, there do not appear to be toxic effects associated with any of the three proteins. A 14 day observation period after dosing did not reveal any abnormalities associated with injection of any of the proteins.
CLASSIFICATION: ACCEPTABLE

MRID 447343-03: All animals appeared healthy, survived to termination of the study, and generally gained weight. No significant differences were seen in the parameters measured for clinical chemistry or hematology. The pathologist reported increased leanness in high dose treated mice seen in both the superficial tissues and abdominal viscera (10/12 animals). The hearts in the treated groups were also noted as having surface hemorrhages in 5/12 and 9/12 animals in the low and high dose Cry9C treatments, respectively. No unusual findings were made of any of the histopathology sections examined. The immunocytological examination of the GI tract found no binding of the the Cry9C protein to villi or enterocytes lining the crypts of both the large and small intestines. Lymphatic tissue of the intestines (i.e., Peyer's patches), the spleen, submandular glands, mesenteric

496

lymph nodes and thymus were all normal upon microscopic examination. Western blot analysis of fecal contents did find Cry9C in a degraded form (55kDa). There was an apparent change in the fat content of the high Cry9C dose group which was seen in a subjective determination of decreased fat pad size. The 28 day (and only) urinalysis of the high dose Cry9C group also indicated an elevated ketone level. Both these findings indicate some perturbation in the fat metabolism of the high dose group which has unclear toxicological significance.

CLASSIFICATION: Acceptable. The dietary exposure study was not required for this active ingredient.

MRID 447343-04: Cry9C protein expression data was generate from whole and processed corn samples collected from transgenic and non-transgenic corn plants. The data presented in Tables 2, 3 & 4 indicate the amount of the PAT and Cry9C proteins present in the respective parts of the corn and corn products. Table 4 provides relative information regarding the amounts of each of these proteins, and their amounts as a percentage of total proteins in the representative materials. Overall, based upon the data provided, these proteins are present at a maximum percentage of 0.0685% (dry mill - solvent extract germ), representing a relative small amount of total protein.

However, these data are somewhat questionable due to the levels of proteins found in the control samples grown in Illinois. It is certainly odd that both proteins are found in many of the control samples. It is possible that these results are simply the result of contamination of the control corn samples either in the field, or during the processing phase of the study.. The validation assay was carried out using samples from a control plot of Glufosinate resistant corn grown in North Carolina (1997). The data and analysis from this study appear to be adequate, however, because the control was a different line of corn, grown in a different state under different (unidentified) growth conditions, these data can also be considered questionable in their relevance to this study. In addition, because they do not address the issue of why the control samples gave positive results for the proteins in question, they do not appear to resolve the issue of the Illinois-grown controls.

As they are presented, the overall numbers do support the suggestion by AgrEvo that the Cry9C and PAT proteins represent a relatively small amount of the total proteins found in the transgenic plants. However, this is based upon the assumption that the titers of the proteins provided in this report are accurate. Because the control samples did show positive signals for each proteins, the accuracy of these numbers is questionable.

CLASSIFICATION: SUPPLEMENTAL. This submission can be upgraded to ACCEPTABLE with submission an adequate explanation for why the control samples also showed positive ELISA results for the PAT and Cry9C proteins, or supplemental data to address this issue.

MRID 447343-05: All three batches of Lys mutant Cry9C were shown by SDS-PAGE and western blot to be substantially made up of Cry9C protein compared to the 100% standard provided by PGS. Greater than 90% of each preparation had the 68 kDa band as the major component with the majority of the remaining contaminant being the 55 kDa degradation form. The three batches of Lys mutant Cry9C either from *E.coli* (batches I & II) or *B. thuringiensis* (batch III) were shown to be substantially resistant to degradation after 2 hours by either SGF or SIF. When the Cry9C samples

were subject to heat (either room temperature 20°C or 90°C) for extended periods the protein appear to be stable as the protein banding pattern in SDS-PAGE gels is unaltered. The presence of a tomato matrix does not appear to affect this heat stability. However, the company has presented an isoelectric focusing gel which purports to show a loss of detectable protein forms after 30 to 60 minutes. The data for bioavailability of Cry9C protein was monitored by the indirect double antibody sandwich ELISA previously described which had a detection limit of 2.0 ngCry9C/ml in serum with a recovery rate of 85±5%. Previous work with Cry1Ab5 is also presented and that ELISA had a detection limit of 5.0 ngCry1Ab5/ml with a recovery rate of 55±5%. The animals were dosed with a range of Cry9C from 2.6 to 298 mg/kg bodyweight by gastric intubation. All the dosed animals appeared and behaved normally, survived to sacrifice and displayed no treatment related adverse effects. No ELISA positive Cry9C protein or fragments were detected in blood samples taken from rats at the 2.6mg/kg dose. At the higher dose rates, Cry9C ELISA values were positive giving a calculated value of between 5 and 15 ng Cry9C/ml in serum. The absorption rate was not affected by dose amount or presence of a food matrix. The percentage of bioavailable Cry9C was calculated to range from 0.0006% (42mg/kg) to 0.0002% (298mg/kg) of the dose administered. More importantly, an SDS-PAGE/western blot confirmation of the sera that were ELISA positive for the presence of Cry9C protein showed that only one of the rats had serum with confirmed Cry9C protein. This rat (#548) had a faint Cry9C positive band, all the other sera had no detectable Cry9C reactivities. No interfering compounds were found in rat serum or BSA. The positive signals were were attributed to cross-reaction with Cry9C like components present in both control and Bt-treated animals since there were no significant differences between the 2-D electrophoresis or ECL results. This data indicating cross reaction was not presented.
CLASSIFICATION: Acceptable.

6996

DATA EVALUATION REPORT

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

Secondary Reviewer: Sheryl Reilly, Ph.D., Immunologist, BPPD

STUDY TYPE:	Food Allergenicity-Brown Norway Rat Model
MRID NO:	447140-02
CHEMICAL NO:	006466
TEST MATERIAL:	Cry9C protein from *Bacillus thuringiensis* ssp. *tolworthi*
STUDY NO:	Contract No. EU AIR3 CT94-2311
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	BIBRA International, Carshalton, Surrey, UK
TITLE OF REPORT:	Development of New Methods for Safety Evaluation of Transgenic Food Crops
AUTHOR:	Sally Van Wert, Ph.D.; Dr. Clive Meredith
STUDY COMPLETED:	20 November 1998
CONCLUSION:	The results of intraperitoneal injection of corn powder extracts into BN rats indicate that both the control and transgenic corn powders are able to induce IgE or reagininc antibody responses by the PCA assay. The use of corn powder immunogen decreases the rate of the immune response to the Cry9C protein compared to the bacterial preparation. However, the lowest responding dose for Cry9C was similar for the two preparations (between 0.1 and 0.4 µg Cry9C). The control challenge test with the heterologous antigen of control corn powder or transgenic corn powder in the day 42 sera samples indicate that there was significant reactivity form the corn portion of the extracts themselves in the PCA assay. It is unclear, given this background reactivity, how suppositions can be made about the reactivity of the Cry9C protein alone. The PCA results from oral sensitization with ovalbumin II, control corn extract, bacterial Cry9C and transgenic corn (apparently supplemented with bacterial Cry9C) indicated that an IgE or reagin antibody response was elicited in naïve Sprague-Dawley rats. Ovalbumin produced a low frequency of responders and a weak dose response between the 5.0 and 50.0 mg/kg dose levels on days 28 through 42. The control corn also produced a positive oral sensization response, but this was only examined at the 50mg/kg dose. Oral dosing with bacterial Cry9C gave a positive PCA response as did the Cry9C amended transgenic corn extract. The frequency of response to bacterial Cry9C began to diminish in day 42 sera. The Cry9C amended transgenic corn had a higher frequency of responders and the frequency remained high on day 42 PCA response. Western blot analysis indicated that Cry9C protein bands could be recognized in the rat sera from both exposure routes.
CLASSIFICATION:	Supplemental. A more detailed description of the materials and methods, especially how the PCA system was utilized in these experiments, is needed. Specifically: 1) how long after the serum

1911

injections were the challenge antigen injections done; 2) what was the concentration of Cry9C in the bacterial and corn plant powder preparations; 3) The source of the test animals and their care during the test were not described; 4) How many naïve Sprague-Dawley rats were used for each serum tested; 5) What were the individual animal responses; 6) The original gel for the western blot assay should be provided.

STUDY DESIGN

The brown Norway (BN) rat model for the detection of food allergens has been used to determine the potential of the Cry9C protein to induce a food sensitization response. The model has not been validated and the study was not conducted according to the 40 CFR 160 GLP standards. In addition there were results from an initial antibody production study to develop a western blot detection system and a sensitization assay with both Cry1Ab5 and Cry9C proteins reported but not described.

Test material: Bacterially produced Cry1Ab5 and Cry9C were provided by Plant Genetic Systems, Ghent, Belgium. There were four batches of the bacterial produced Cry9C protein extracts provided at the following amounts: 1) 20mg of a white powder; 2) 1.6g of a buff powder; 3) 60 mg of a white powder and 4) 10g of a buff powder. There was no analysis of these bacterial powders for purity or Cry9C content. Samples of corn powder extract from both non-transgenic plants and plants expressing the Cry9C protein were also provided by PGS. These extracts are called "whole corn plant powder." There were also two batches of corn powder provided: 1) the transgenic Cry9C corn powder with 4% Cry9C (TPPE-351-0197) and the non-transgenic corn powder (TPPE-351-0197c) free of Cry9C; 2) the transgenic corn powder with 4.7% and the non-transgenic corn with 0.05% detectable Cry9C. There was no indication that the testing lab did these concentration studies. Ovalbumin grade II was obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K. The solutions used in these experiments were prepared by dispersing them in phosphate buffered saline, mixing, homogenizing and separating the aqueous fraction by centrifugation.

Test animals: Groups of ten brown Norway (BN) rats were used for the initial study with intraperitoneal injection for the sensitization phase. Groups of eight BN rats were used for the oral sensitization phase. Naïve Sprague-Dawley rats were used for the passive cutaneous anaphylaxis (PCA) tests with sera obtained from the BN rats. No details were given as to where the animals used in these studies were obtained, their general health nor how they were maintained during the test.

Test methods:

Passive Cutaneous Anaphylaxis: The basis of these studies is the PCA assay where serum from a sensitized animal is subcutaneously injected into a naïve animal. Any IgE (also termed reagin) present in the injected serum will bind mast cells present in the naïve animal. Subsequently, the alleged allergen together with a protein binding dye is intravenously injected into the tail vein of the naïve animals. If a mast cell degranulation reaction occurs, the area around the subdermal injection site will become infiltrated with dye as the blood vessels near the site become more permeable due to the histamine and other modulators released during degranulation. The intensity of the reaction is expressed as the area of dye infiltrated skin near the subcutaneous injection site. This

hypersensitivity phenomenon was originally described in 1921 as is sometimes referred to as the Prausnitz and Kustner reaction.

Sensitization by intraperitoneal injection: The initial study was performed with groups of ten BN rats injected with 1.0, 10 or 100µg of transgenic or non-transgenic corn powder. The extract was mixed with 10 mg of carrageenan in 1 ml of saline. Carrageenan is reported to be a good adjuvant for the IgE response in BN rats. The amount of Cry9C protein in these extracts was 0.04, 0.4 or 4.0 µg. It is unclear from the description whether there was more than one injection prior to the PCA assays. Blood samples were taken from the treated BN rats at 14, 21, 28, 35 and 42 days. These serum samples we tested for IgE response with the PCA assay using naïve Sprague-Dawley rats. Cross-reactivity was tested with sera taken on day 42 as follows: serum from the Cry9C corn powder sensitized individuals was tested against a challenge with non-transgenic corn powder and the non-transgenic corn powder sensitized individuals were challenged with transgenic corn powder.

Sensitization by the oral route: This study was done by exposing groups of eight BN rats to four different antigen treatments: 1) bacterial Cry9C (batch 4); 2) ovalbumin II; 3) control corn powder (TPPE-351-0197c, batch 2) or 4) transgenic corn powder (TPPE-351-0197, batch 2). The dosing was done with 0.5ml/100g bodyweight twice a week for six weeks. No description of the composition of the dosing solutions was given nor could it be determined if dosing was done by gavage or drinking water. However, data tables indicate a dose range of 1.0, 2.0 and 10.0mg/ml or 5, 10 and 50mg/kg bodyweight for the ovalbumin II and Cry9C by oral sensitization. The BN rats were also given intraperitoneal injections of carrageenan (1mg in 1ml of saline) once a week for six weeks prior to the oral sensitization phase. Blood samples were taken from the rats at weekly intervals after the sensitization (days 14, 21, 28, 35 and 42). The PCA tests were done with naïve Sprague-Dawley rats which included as controls challenges with non-transgenic corn or Cry9C amended corn extracts.

RESULTS AND DISCUSSION

The reported results of the studies are attached as an appendix to this review. The experimental method for some of the data reported in the tables were not described in the materials and methods sections. Tables 1 & 2 indicate that both Cry1Ab5 and Cry9C are capable of inducing an IgE or reagininc antibody response in BN rats following intraperitoneal injection with bacterial preparations by the PCA assay. The authors claim that Cry9C has a lower response threshold since a higher frequency of responding BN rats were observed than for Cry1Ab5.

In tables 3 & 4, the results of intraperitoneal injection of corn powder extracts into BN rats indicate that both the control and transgenic corn powders are able to induce IgE or reagininc antibody responses by the PCA assay. The authors again claim that the response for Cry9C was greater due to the higher number of responders and the earlier onset of IgE response.

Comparing the serum response between tables 2 & 4 indicates the use of corn powder immunogen decreases the rate of the immune response to the Cry9C protein compared to the bacterial preparation (day 21 compared to day 14). However, the lowest responding dose for Cry9C was similar for the two preparations (between 0.1 and 0.4 µg Cry9C). The control challenge test with the heterologus antigen of control corn powder or transgenic corn powder in the day 42 sera samples indicate that

3911

there was significant reactivity form the corn portion of the extracts themselves in the PCA assay. It is unclear, given this background reactivity, how suppositions can be made about the reactivity of the Cry9C protein alone.

In table 5, sera from the control corn powder intraperitoneal injection sensitized animals did not react with bacterial Cry9C. The response of transgenic corn sensitized serum to non-transgenic corn, transgenic corn or bacterial Cry9C extracts was similar. The response of bacterial Cry9C sensitized sera to different batches of bacterial Cry9C were positive and it appeared that there were higher concentrations of the Cry9C protein present in batches 2 and 3.

In tables 6 & 7, the PCA results from oral sensitization with ovalbumin II, control corn extract, bacterial Cry9C and transgenic corn (apparently supplemented with bacterial Cry9C) indicated that oral exposure elicited an IgE or reagin antibody response in the naïve Sprague-Dawley rats. The ovalbumin sensitized serum had a low frequency of responders and showed a weak dose response between the 5.0 and 50.0 mg/kg dose levels on days 28 through 42. The control corn also gave a positive oral sensization response but this was examined only at a single dose (50mg/kg). Oral dosing with bacterial Cry9C gave a positive PCA response as did the Cry9C amended transgenic corn extract. The frequency of response to bacterial Cry9C began to diminish in day 42 sera. The Cry9C amended transgenic corn produced a higher frequency of responders, which remained high on day 42.

Finally, a western blot analysis of sensitized rat sera from both the oral and intraperitoneal exposure methods was presented (figure 4). Reactive bands were observed at several locations including the 66kDa band expected for Cry9C, another band at approximately 100kda and also several lower molecular weight bands. There are also positive bands at these same regions in the control non-transgenic corn extracts. One of the problems with interpreting this figure is that it has been highly manipulated by the digitized presentation format. The Coomassie blue stained lanes are very weakly stained which is unlikely in a plant extract with any significant protein content. A matchup between the low and the high molecular weight markers on either end of the gel is not possible. Overall, the bands, while separated in the presentation format used, indicate that there was probably a smile in the banding pattern which is extremely difficult to evaluate if the original gel is not presented.

REVIEWER COMMENT

It is unclear from the studies or the literature provided that the idea of "relative potency" for proteins to be food allergens is a valid concept. That a protein can be shown to induce an IgE response is a first step towards identifying food allergen potential. The fact that many humans are IgE serum positive for a protein that does not correlate with an expressed allergy in these people would confound that assumption. The detection of mast cell degranulation by the PCA system would be a next step. However, it is not apparent from the provided materials and methods description that appropriate measures were taken to verify the PCA test. According to the cited literature, the antigen/dye injection was done 24 hours after subcutaneous serum injection. This is less than the normal 48-72 hours required to allow diffusion of cross reacting IgG which could also cause mast cell degranulation and give a false positive reaction. Another proof against false positive reactions would be the heat treatment of test serum to inactivate IgE and confirm that the response was due to antigen specific IgE. It would have been clearer to include proteins that have not been implicated

4911

as food allergens (i.e., bovine serum albumin) to test the model for false positives. According to other information included in the data package, standard protocol in the PCA system is to dilute out the test sera to determine the level of serum IgE reactivity, not to note the frequency of responders and area of the dye extravasation.

An independent determination of the concentration of the Cry9C protein in the bacterial preparations tested is essential. The oral sensitization schedule is unclear as to when the carrageenan intraperitoneal adjuvant was stopped and when oral dosing with the various antigens began. The western blot data presented needs a materials and methods section to describe the process and sera used and inclusion of the original gel or membrane in order to render any conclusions about serum reactivity.

5011

Appendix to BN RAT STUDY

Table 1. Reaginic antibody response in those animals developing a response following exposure to Cry1ab5 by the intraperitoneal route.

Day		Area of dye extravasation mm²				
		Cry1ab5 μg/ml				
		100	10	1.0	0.1	0.01
14	Mean	302	313	242	0	0
	SD	85.0	116.0	91.3	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8
21	Mean	307	225	245	0	0
	SD	92.2	109.1	66.4	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8
28	Mean	314	229	197	0	0
	SD	88.3	49.0	28.5	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8
35	Mean	302	210	189	0	0
	SD	91.2	54.1	13.9	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8
42	Mean	260	222	195	0	0
	SD	77.9	46.0	12.0	0.0	0.0
	Responders	7/8	4/8	4/8	0/8	0/8

Table 2. Reaginic antibody response in those animals developing a response following exposure to Cry9c by the intraperitoneal route.

Day		Area of dye extravasation mm²				
		Cry9c μg/ml				
		100	10	1.0	0.1	0.01
14	Mean	214	319	253	217	0
	SD	82.7	82.3	30.6	128.5	0.0
	Responders	4/8	7/8	8/8	6/8	0/8
21	Mean	249	297	290	206	0
	SD	66.5	53.3	60.0	94.6	0.0
	Responders	6/8	7/8	8/8	6/8	0/8
28	Mean	216	260	264	232	0
	SD	22.4	68.7	77.9	103.8	0.0
	Responders	6/8	7/8	8/8	6/8	0/8
35	Mean	128	126	229	161	0
	SD	25.2	32.0	102.0	24.6	0.0
	Responders	6/8	7/8	8/8	6/8	0/8
42	Mean	138	201	245	182	0
	SD	50.9	80.3	114.0	120.0	0.0
	Responders	6/8	7/8	8/8	6/8	0/8

Table 3. Reaginic antibody responses in those animals developing a response following exposure to Control corn powder extract by the intraperitoneal route.

Control corn powder extract μg		Area of dye extravasation mm²					
		Day					
		14	21	28	35	42	42*
1	Mean	0	0	0	333	209	284
	SD	0.0	0.0	0.0	0.0	106.8	0.0
	Responders	0/10	0/10	0/9	1/9	2/10	2/8
10	Mean	0	0	104	128	174	225
	SD	0.0	0.0	80.4	140.7	136.4	68.1
	Responders	0/10	0/10	3/10	2/10	4/10	4/10
100	Mean	0	28	127	151	127	167
	SD	0.0	0.0	29.9	140.7	105.8	50.7
	Responders	0/10	1/10	3/10	3/10	4/10	6/10

PCA challenge carried out using control corn powder extract, * PCA challenge carried out using transgenic corn powder extract

Table 4. Reaginic antibody responses in those animals developing a response following exposure to transgenic corn powder exract by the intraperitoneal route.

Transgenic corn powder extract μg (Cry9C μg)		Area of dye extravasation mm²					
		Day					
		14	21	28	35	42	42*
1 (0.04)	Mean	0	0	0	0	0	0
	SD	0.0	0.0	0.0	0.0	0.0	0.0
	Responders	0/10	0/10	0/10	0/10	0/10	0/10
10 (0.4)	Mean	0	150	136	180	138	145
	SD	0.0	69.9	74.4	47.4	86.3	45.3
	Responders	0/10	6/10	5/10	6/10	6/10	2/10
100 (4.0)	Mean	0	195	257	164	206	125
	SD	0.0	67.8	76.4	31.6	27.7	92.6
	Responders	0/10	8/10	8/10	8/10	7/10	6/10

PCA challenge carried out using bacterial Cry9C, * PCA challenge carried out using control corn powder extract

Table 5. Examination of specificity of PCA responses to Cry9C in sera obtained following exposure to different batches of bacterial Cry9C, control corn extract and transgenic corn extract by the intraperitoneal routes.

		ADE mm²		
Treatment		PCA Challenge material		
Control corn powder extract (1)		Control corn	Transgenic corn	Cry9C (3)
An.No	9.56	255	201	0
	9.61	64	33	0
	9.62	64	154	0
	9.65	95	227	0
		120 95	154 96	
Transgenic corn powder extract (1)		Control corn	Cry9C (3)	Cry9C (3)
An.No.	9.90	177	177	177
	9.91	177	113	201
	9.96	284	227	201
	9.98	85	201	177
		181 81	180	13.9
Cry9c batch 1		Cry9C (1)	Cry9C (2)	Cry9C (3)
An.No.	7.32	201	531	573
	7.37	177	380	416
	7.40	177	346	346
		185 13.9	419 93.5	445 116.2

Cry9C (total quantity received at BIBRA from PGS)

batch 1 20 mg
batch 2 1.6 g
batch 3 60 mg
batch 4 10 g

Table 6. Reaginic antibody response in those animals developing a response following exposure to Ovalbumin II or control corn by the oral route.

		Area of dye extravasation mm²			
		Ovalbumin II			Control Corn *
Day	mg/ml (mg/Kg)	10.0 (50)	2.0 (10)	1.0 (5)	10.0 (50)
14	Mean	0	0	0	0
	SD	0.0	0.0	0.0	0.0
	Responders	0/8	0/8	0/8	0/8
21	Mean	0	0	0	33
	SD	0.0	0.0	0.0	0.0
	Responders	0/8	4/8	0/8	1/8
28	Mean	184	0	0	167
	SD	100.4	0.0	0.0	48.1
	Responders	2/8	0/8	0/8	2/8
35	Mean	347	197	177	195
	SD	46.7	111.2	0.0	148.7
	Responders	2/8	2/8	1/8	5/8
42	Mean	226	173	201	166
	SD	154.8	64.8	0.0	81.0
	Responders	4/8	3/8	1/8	7/8

* PCA challenge with control corn

Table 7. Reaginic antibody response in those animals developing a response following exposure to Cry9C or transgenic corn by the oral route.

		Area of dye extravasation mm²			
		Cry9C			Transgenic Corn : Cry9C **
Day	mg/ml (mg/Kg)	10.0 (50)	2.0 (10)	1.0 (5)	10.0 : 0.045 (50 : 0.225)
14	Mean	0	0	0	491
	SD	0.0	0.0	0.0	0.0
	Responders	0/8	0/8	0/8	1/8
21	Mean	124	227	0	222
	SD	91.8	0.0	0.0	63.8
	Responders	3/8	1/8	0/8	7/8
28	Mean	283	339	270	375
	SD	77.6	199.0	83.9	209.2
	Responders	6/8	6/8	6/8	7/8
35	Mean	230	382	331	287
	SD	145.2	234.9	146.6	108.1
	Responders	4/8	6/8	6/8	8/8
42	Mean	230	227	255	264
	SD	0.0	0.0	0.0	82.9
	Responders	1/8	1/8	1/8	7/8

** PCA Challenge with Cry9C

Table 8. Examination of specificity of PCA responses to Cry9C in sera obtained following exposure to different batches of bacterial Cry9C, control corn extract and transgenic corn extract by the oral route.

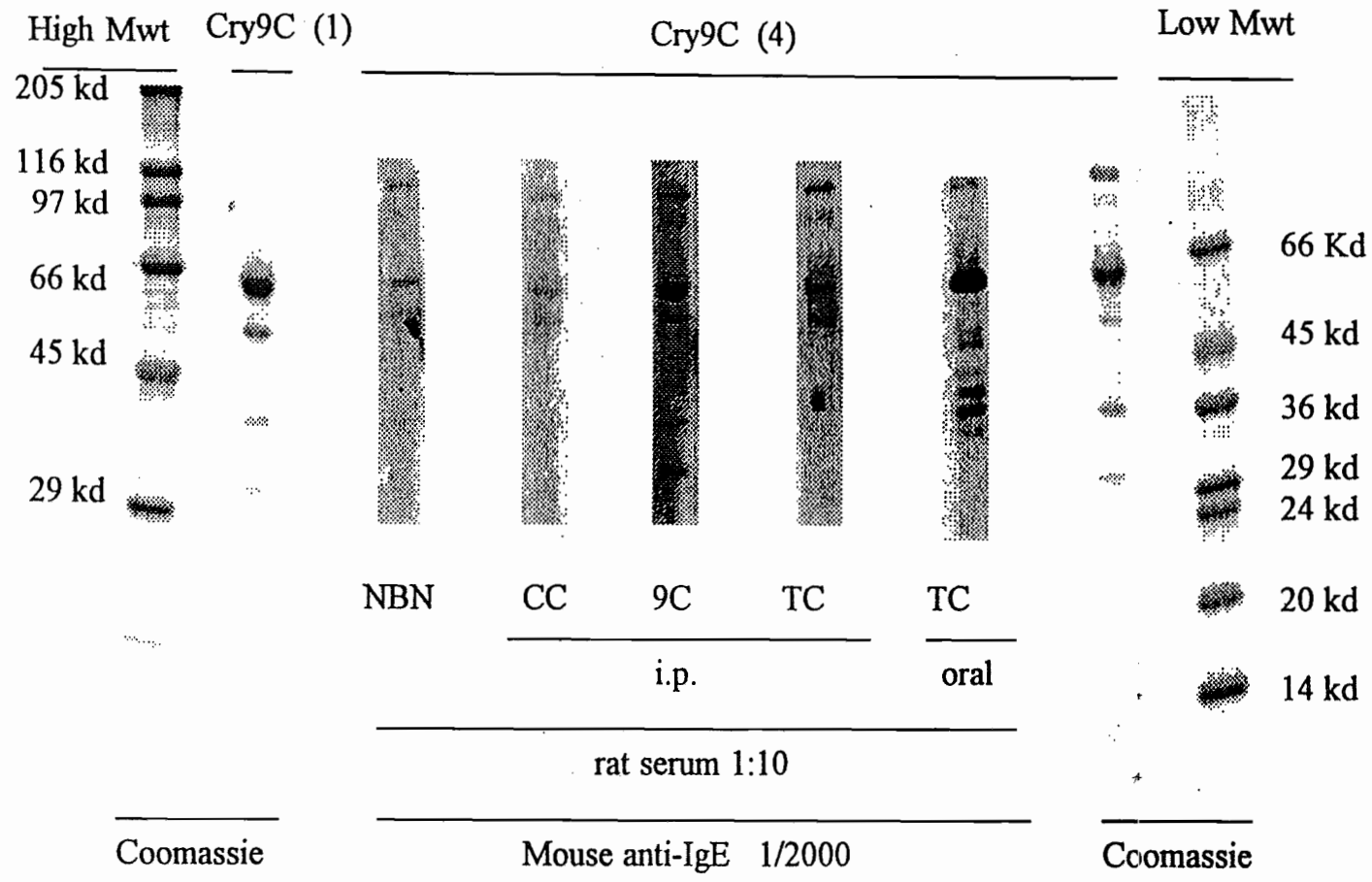
		ADE mm^2	
Treatment		PCA challenge material	
Control corn powder extract (2)		Control corn	Cry9C (3)
An.No	11.49	177	312
	11.53	227	284
	11.54	201	255
	11.55	255	255

Transgenic corn powder extract (2)		Cry9C (4)	Cry9C (3)
An.No.	11.57	177	300
	11.58	227	255
	11.59	284	314
	11.60	314	416
	11.61	227	154
	11.62	419	255
	11.63	201	154
	11.64	0	0

Cry9C (total quantity received at BIBRA from PGS)

batch 1 20 mg
batch 2 1.6 g
batch 3 60 mg
batch 4 10 g

Figure 4.


High Mwt
Cry9C (1)
Cry9C (4)
Low Mwt
205 kd
116 kd
97 kd
66 kd
45 kd
29 kd
66 Kd
45 kd
36 kd
29 kd
24 kd
20 kd
14 kd
NBN
CC
9C
TC
TC
i.p.
oral
rat serum 1:10
Coomassie
Mouse anti-IgE 1/2000
Coomassie

04 / 0

A

DATA EVALUATION REPORT

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

Secondary Reviewer: Michael Watson, Ph.D., Plant Pathologist, BPPD MW

(1P)

STUDY TYPE:	Hypersensitivity Incidence Reporting
MRID NO:	447140-03
CHEMICAL NO:	006466
TEST MATERIAL:	Cry9C protein from *Bacillus thuringiensis* ssp. *tolworthi*
STUDY NO:	none assigned
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	Garst Seeds, Slater, IA
TITLE OF REPORT:	Occupational Exposure of StarLink™ Corn: Garst Seeds, 1996-1998
AUTHOR:	Sally Van Wert, Ph.D.
STUDY COMPLETED:	20 November 1998
CONCLUSION:	The testimonial letters submitted by employees of the Garst Seed Company indicate that 1980 people with considerable direct exposure to corn seed and plant parts including tassels and pollen have not experienced adverse or allergic responses they could directly attribute to exposure to the Cry9C protein in StarLink™ corn.
CLASSIFICATION:	Acceptable. The company is reminded that they are still responsible to report any incidents of hypersensitivity or other adverse effects they know has resulted from exposure to the Cry9C protein.

STUDY DESIGN

There is no method for this survey and the work was not done according to GLP. This is simply an accounting by the Garst Seed Company of their employees who have worked with the StarLink™ corn expressing Cry9C protein and their statements to the effect that none of them have reported adverse effects from their exposure.

The report includes thirty-four testimonial letters from people involved in various aspects of hybrid corn development such as bagging corn seed, scouting for insect damage, de-tasseling and hand pollinating. The thirty-four letters represent the responses from 1980 people. The vast majority of the people responding (1946) were found in ten letters from field production managers. The ten field production managers supervised 1936 people and reported by a form letter that they had not observed any allergic reactions to the StarLink™ corn used in hybrid production. Within the form letter is a disclaimer that "Each year we have employees that work in our fields that will have reactions to pollen dust. The reactions that we have had this year are considered normal and can not be attributed to any particular field or inbred." Also within the group of letter writers were two individuals who are atopic and reported that exposure to StarLink™ corn has not increased their level of hypersensitivity.

191

DATA EVALUATION REPORT

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

Secondary Reviewer: Michael Watson, Ph.D., Plant Pathologist, BPPD

STUDY TYPE:	30-Day Dietary Toxicity in the Rodent
MRID NO:	447343-03
CHEMICAL NO:	006466
TEST MATERIAL:	Cry9C protein from *Bacillus thuringiensis* ssp. *tolworthi*
STUDY NO:	RIKILT-DLO No. 71.113.04.D97.4
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	RIKILT-DLO, Netherlands
TITLE OF REPORT:	Mouse Short-Term (30-Day) Dietary Toxicity Study with the Protein Cry9C
AUTHOR:	Sally Van Wert, Ph.D.; Dr. H.P.J.M. Noteborn
STUDY COMPLETED:	4 September 1998
CONCLUSION:	All animals appeared healthy, survived to termination of the study, and generally gained weight. No significant differences were seen in the parameters measured for clinical chemistry or hematology. The pathologist reported increased leanness in high dose treated mice seen in both the superficial tissues and abdominal viscera (10/12 animals). The hearts in the treated groups were also noted as having surface hemorrhages in 5/12 and 9/12 animals in the low and high dose Cry9C treatments, respectively. No unusual findings were made of any of the histopathology sections examined. The immunocytological examination of the GI tract found no binding of the the Cry9C protein to villi or enterocytes lining the crypts of both the large and small intestines. Lymphatic tissue of the intestines (i.e., Peyer's patches), the spleen, submandular glands, mesenteric lymph nodes and thymus were all normal upon microscopic examination. Western blot analysis of fecal contents did find Cry9C in a degraded form (55kDa). There was an apparent change in the fat content of the high Cry9C dose group which was seen in a subjective determination of decreased fat pad size. The 28 day (and only) urinalysis of the high dose Cry9C group also indicated an elevated ketone level. Both these findings indicate some perturbation in the fat metabolism of the high dose group which has unclear toxicological significance.
CLASSIFICATION:	Acceptable. The dietary exposure study was not required for this active ingredient.

STUDY DESIGN

This study was not done according to the GLP standards found in 40CFR160 or the OECD Council Decision C(81)30 of 12 May 1981. The report is also being claimed as confidential by RIKILT-DLO but not by AgrEvo. AgrEvo's statement on data confidentiality also states that AgrEvo's claim

supercedes all other confidentiality statements in the report. The study was an oral 28-day exposure to Cry9C protein through drinking water.

Test material: The test substance was a purified trypsinized form of the Cry9C protein (Lys164Arg) as expressed in a *Bacillus* strain with the cry9C.PGS1a gene. The test substance was provided by Plant Genetic Systems in two lyophilized batches which were run on SDS-PAGE to confirm their composition/purity. The protein content was determined by a BCA Protein Assay (Pierce Chemicals, Rockford, Il) with BSA as the standard.

Test animals: Forty-three ♀ mice (NMRI outbred) were obtained from a specific pathogen free colony at Harlan Germany and weighed between 27.6 and 32.8g at receipt. After isolation and acclimatization, thirty-six mice were chosen for use in the study based on their weight gains from receipt and bodyweight variation being less than 15% of the 29.0g mean. The animals were identified by ear tag and kept two per cage during the study. For two days over the 28-day test period, the animals were kept in metabolic cages. Otherwise they were maintained in the Central Experimental Animal Accommodation under standard conditions (12 hours artificial light/dark at 21±1 °C with a controlled humidity of 55±10 % which only rose during the weekly cleaning to 80-90 % RH for approximately one hour). The mice received the Institute's basal rodent diet (RMH-B) and liquid *ad libitum*. Tap water (supplemented with test substance for the treated groups) was provided in glass bottles which were refilled daily with fresh solution.

Test methods:
Dosing: The test substance was provided to the mice in their water. The test substance doses amounts are based on a daily consumption of 5.0 ml water/30.0 gm mouse. The provided solutions were at 0.21 or 2.1 gm Cry9C/l for a calculated dosing amount of 35 or 350 mg/kg. The dosing was begun October 6 and 7, 1997 and terminated November 4 and 5, 1997. Fifteen samples from the drinking water bottles were taken throughout the dosing regime after standing at room temperature for 24 hours. These samples were subjected to a western blot analysis to confirm the composition and stability of Cry9C in the dosing solution. The weekly group mean of the Cry9C intake was monitored by tracking body weight and liquid consumption. The individual animals were weighed weekly, water consumption per cage of two animals was recorded daily and the food consumption monitored by weight weekly. Feed utilization efficiency (%) was calculated by tracking the group mean body weight gain (g/animal/day) divided by the group mean food consumption (g/animal/day) x 100.

Clinical Signs and Biochemical Monitoring: The general condition and behavior of the animals was examined twice daily on weekdays and once daily on the weekends. On day 5 before the start of the dosing and on day 29 blood samples were collected from the orbital sinus and subjected to routine hematology including the following: hemoglobin, red and white blood cell count, hematocrit, mean cell volume, mean cell hemoglobin, mean cell hemoglobin concentration, and reticulocyte, neutrophil, lymphocyte, monocyte, eosinophil and basophil cell counts. On day 30 additional blood chemistry was performed on samples taken examining the following: alkaline phosphatase, total protein, albumin, bilirubin, urea nitrogen, creatine, glucose, cholesterol, calcium, serum glutamic oxaloacetic transaminase and hemoglobin. On day 28 the mice were placed in metabolic cages to

collect urine for routine urinalysis which included: volume, pH, protein, glucose, blood, ketone, and bilirubin content.

An ELISA was run on the blood serum and 24 hour feces samples to determine Cry9C levels present. The 24 hour feces sample taken during the metabolic caging was frozen, diluted 1:1 in 50mM TrisHCL (pH7.3) with 1.0mM PMSF and 2.0mM EDTA. The extract was allowed to sit for one hour on ice before the supernatant was diluted 1:1 with 10mM Tris-HCl (pH8.0) with 1.0mM EDTA, 20% glycerol, 5% SDS, 2% dithiothreitol and 0.2% bromophenol blue. This solution was then boiled for 5 minutes and stored at -40°C until western blot analysis was performed.

After sacrifice on day 30, all animals received a gross pathological examination with a bone marrow smear of the femur being made at the same time. The weights of the following organs were recorded: adrenal, brain, heart, kidneys, liver, lungs, ovaries, spleen and thymus. Samples of the following organs were taken and preserved in formalin: brain, caecum, colon, femur with joint, heart, kidneys, liver, lungs, esophagus, ovaries, pancreas, rectum, thigh muscle, skin, small intestines, spleen, stomach, thymus, urinary bladder, uterus and any other tissue with gross macroscopic abnormalities. Sections of the following tissues were made, stained in eosin/Haematoxylon and examined by a pathologist: bladder, mesenteric lymph nodes, brain and spinal cord, caecum, colon, duodenum, heart and thyroid/trachea, ileum, jejunum with Peyer's patches, kidneys and adrenal glands, liver and lungs, esophagus and stomach, ovaries, rectum, spleen and thymus and uterus.

RESULTS AND DISCUSSION

Test material

The test material analyzed by PGS indicated that Batch I consisted of 100% Lys mutant Cry9C protein (MW 68kDa) and Batch II, approximately 70%. Both batches were approximately 1.1g in size. The remaining 30% of Batch II was said to contain untrypsinized protoxin, inactivated trypsin and inactivated Cry9C degradation products. RIKILT-DLO repeated a western blot and scanning densitometry analysis of Batch II to verify that 70-75% of the material was indeed Cry9C (68kDa). The testing lab also verified that the test substance was stable in solution for one day with spontaneous degradation to the 55kDa form.

Dose verification and food consumption

The uptake of the test substance was monitored by the daily water consumption which showed no differences among the treated groups and the controls. Therefore, the two treatment groups, based on average daily consumption of test substance amended water, received 33.3 $\pm$0.3mg or 328.0$\pm$4.0mg Cry9C per kg bodyweight per day. There was no effect on food consumption related to treatment group.

Bodyweights and Clinical Signs

All animals appeared healthy, survived to termination of the study, and generally gained weight. There were transient weight losses which were similar across treatment and control groups. No treatment related effects on body weight could be seen although individual animal data was not provided. No treatment groups gained more than 2.3g (~7% gain) from the initial weight during the course of the study. All animals lost bodyweight beginning day 28 and 29 when the groups were

being held in metabolic cages. Up to that date all the animals had been gaining weight at a similar rate.

Clinical Chemistry, Urinalysis and Hematology

No significant differences were seen in the parameters measured for clinical chemistry or hematology. Calcium and SGOT were not measured due to degradation or treatment of the blood samples. Decreased urinary volume and increased ketone were noted in urine samples from the high dose Cry9C treated animals taken in the metabolic cages. No Cry9C was found in the blood by ELISA.

Autopsy and Histopathology

Minimal common gross pathological findings were noted among the treatment groups such as pale kidneys, spotted spleen and lungs. The pathologist reported increased leanness in high dose treated mice seen in both the superficial tissues and abdominal viscera (10/12 animals). The hearts in the treated groups were also noted as having surface hemorrhages in 5/12 and 9/12 animals in the low and high dose Cry9C treatments, respectively. No unusual findings were made of any of the histopathology sections examined. The immunocytological examination of the GI tract found no binding of the Cry9C protein to villi or enterocytes lining the crypts of both the large and small intestines. Lymphatic tissue of the intestines (i.e., Peyer's patches), the spleen, submandular glands, mesenteric lymph nodes and thymus were all normal upon microscopic examination. Western blot analysis of fecal contents did find Cry9C in a degraded form (55kDa).

There was an apparent change in the fat content of the high Cry9C dose group which was seen in a subjective determination of decreased fat pad size. The 28 day (and only) urinalysis of the high dose Cry9C group also indicated an elevated ketone level. Both these findings indicate some perturbation in the fat metabolism of the high dose group which has unclear toxicological significance.

DATA EVALUATION REPORT

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

Secondary Reviewer: Michael Watson, Ph.D., Plant Pathologist, BPPD

STUDY TYPE:	*In vitro* Digestibility and Bioavailability in the Rodent
MRID NO:	447343-05
CHEMICAL NO:	006466
TEST MATERIAL:	Cry9C protein from *Bacillus thuringiensis* ssp. *tolworthi*
STUDY NO:	RIKILT-DLO No. 71.113.04.D97.2-3
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	RIKILT-DLO, Netherlands
TITLE OF REPORT:	Assessment of the Stability to Digestion and Bioavailability of the Lys Mutant Cry9C Protein from *Bacillus thuringiensis* serovar *Tolworthi*
AUTHOR:	Sally Van Wert, Ph.D.; Dr. H.P.J.M. Noteborn
STUDY COMPLETED:	16 September 1998
CONCLUSION:	All three batches of Lys mutant Cry9C were shown by SDS-PAGE and western blot to be substantially made up of Cry9C protein compared to the 100% standard provided by PGS. Greater than 90% of each preparation had the 68 kDa band as the major component with the majority of the remaining contaminant being the 55 kDa degradation form. The three batches of Lys mutant Cry9C either from *E.coli* (batches I & II) or *B. thuringiensis* (batch III) were shown to be substantially resistant to degradation after 2 hours by either SGF or SIF. When the Cry9C samples were subjected to heat (either room temperature 20°C or 90°C) for extended periods, the protein appeared to be stable as the protein banding pattern in SDS-PAGE gels was unaltered. The presence of a tomato matrix does not appear to affect this heat stability. However, the company has presented an isoelectric focusing gel which purports to show a loss of detectable protein forms after 30 to 60 minutes. The data for bioavailability of Cry9C protein was monitored by the indirect double antibody sandwich ELISA previously described which had a detection limit of 2.0 ngCry9C/ml in serum with a recovery rate of 85±5%. Previous work with Cry1Ab5 is also presented and that ELISA had a detection limit of 5.0 ngCry1Ab5/ml with a recovery rate of 55±5%. The animals were dosed with a range of Cry9C from 2.6 to 298 mg/kg bodyweight by gastric intubation. All the dosed animals appeared and behaved normally, survived to sacrifice and displayed no treatment related adverse effects. No ELISA positive Cry9C protein or fragments were detected in blood samples taken from rats at the 2.6mg/kg dose. At the higher dose rates, Cry9C ELISA values were positive giving a calculated value of between 5 and 15 ng Cry9C/ml in serum. The absorption rate was not affected by dose amount or presence of a food matrix. The percentage of bioavailable Cry9C

was calculated to range from 0.0006% (42mg/kg) to 0.0002% (298mg/kg) of the dose administered. More importantly, an SDS-PAGE/western blot confirmation of the sera that were ELISA positive for the presence of Cry9C protein showed that only one of the rats had serum with confirmed Cry9C protein. This rat (#548) had a faint Cry9C positive band, all the other sera had no detectable Cry9C reactivities. No interfering compounds were found in rat serum or BSA. The positive signals were were attributed to cross-reaction with Cry9C like components present in both control and Bt-treated animals since there were no significant differences between the 2-D electrophoresis or ECL results. This data indicating cross reaction was not presented.

CLASSIFICATION: Acceptable.

STUDY DESIGN

Examination of the stability of the Lys mutant Cry9C to *in vitro* digestive solutions and *in vivo* bioavailability and mucosal binding of the Lys mutant Cry9C in rats. The study was not conducted according to the GLP standards in 40 CFR 160 or the OECD Council Decision of 12 May 1981. The report is also being claimed as confidential by RIKILT-DLO but not by AgrEvo. AgrEvo's statement on data confidentiality also states that AgrEvo's claim supercedes all other confidentiality statements in the report.

Test materials: Three batches of the Cry9C toxin test substance (EMBL Z37527) was provided by Plant Genetic Systems (Ghent, Belgium). The batches were stated as being 100% pure by western blot analysis. The amounts were stated as 3.0mg/ml buffer; 2.6mg/ml buffer and 1.1g of freeze-dried powder, respectively. Reference toxins Cry1Ab5 (0.7mg/ml) and CryIIIb (2.1mg/ml) were provided by PGS and Metapontum Agrobios (Italy), respectively.

Certain chemical reagents were also required for the performance of these tests. All were reagent grade obtained from commercial sources except for the following special notations: bovine serum albumin fraction V, trypsin inhibitor, porcine pancreatin, goat anti-rabbit IgG-antibody conjugate to alkaline phosphatase, 5,5-dithiobis-2-nitrobenzoic acid (DNTB), acetylCoA from Sigma Chemical, St. Louis, USA. Trypsin and pepsin from porcine gastric mucosa were obtained from Boehringer Mannheim, Ingelheim, Germany. Tetramethylbenzidine and 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) were from KPL, Inc. Gaithersburg, USA. ^{3}H-propranolol and ^{14}C-PEG were from Amersham, UK. Lugol was from Fluka, Switzerland, Diaminobenzidine from Pierce, USA and the deionized water from a MilliQ unit Millipore, Beford, USA.

Test animals: Fifty-two Wu/Wister rats were obtained from a specific pathogen free colony at the Harlan facility in Germany. While being kept in the Central Experimental Animal Accommodation (Agricultural University, Wageninagen, the Netherlands), the rats were checked for overt signs of ill health or anomalies and assigned a unique cage with a card including the animal identification number, cage number, group letter and study number. During the study each animal was assigned a cage maintained in the animal facility at 21±1 °C with a controlled humidity of 55±10%, artificial

light for 12 hours each day (7:70AM to 7:30PM) and supplied with 10 changes of air per hour. The rats had an average bodyweight of 280g at the start of the experiment and were provided food (basal rodent diet, RMH-B; Hope Farms Woerden, the Netherlands) and water *ad libitum* unless some laboratory functions precluded its advisability.

Test methods: **Protein concentration** was determined with a BCA protein assay kit from Pierce using BSA as a standard. Samples were taken from remaining stock solutions that were kept at -40 to -80°C. The samples were also subjected to western blotting to check stability, purity and composition.

***In vitro* digestibility** was done in both simulated gastric (SGF) and intestinal fluids (SIF) prepared as described in the U.S. Pharmacopeia (vol. XXII, p.1788-1789). The SGF was 32mg of pepsin diluted in 10ml of gastric buffer (0.5g NaCl, 1.75ml of 1.0M HCl in 250ml water, pH 2.0) and the SIF was 0.2g pancreatin diluted in 20ml of intestinal fluid (0.17g KH_2PO_4, 4.7ml of 0.2M NaOH in 20ml of water). The proteins were first subjected to pepsin digestion for up to 2 hours at 37°C then to pancreatin activity for up to 4 hours at 37°C. The proteins were added to 1.0ml of SGF or SIF at a concentration of 50-165µg/ml, respectively. At given time points 50µl aliquots were removed, quenched (Fuchs *et al.*, 1993, Bio/Technology 11:1543-1547) and stored at -40°C until the digestive fate of the proteins of interest and the reference standards were assayed. SGF was monitored for activity by an assay which measured the increase in absorbance at 280nm following TCA precipitation of the SGF digested hemoglobin.

Thermostability testing involved diluting protein to a 0.7 to 0.8mg/ml concentration in water, buffer or tomato matrices. Fifty µl samples were exposed to processing temperatures of from 50 up to 90°C for from 5 to 210 minutes. After the heat treatment the samples were immediately put on ice then frozen to -40°C until analyzed further.

Bioavailability testing required the use of rats with indwelling cannula of the vena porta inserted as described in the literature (Remie *et al.*, 1990, Manual of Microsurgery on the Laboratory Rat, Vol. IV, p.213-230, Elsevier) while employing as anesthesia 0.5ml hypnorm (s.c.), 0.5ml of dormicum (i.p.) and 0.04ml of atropin (s.c.) per kg bodyweight. The rats were checked for general condition, clinical signs and behavior twice daily on weekdays or once daily on weekends. The bodyweights of the rats were recorded initially and three times each week during the recovery period. At each weighing, the rats were handled and carefully examined. Approximately three weeks after recovery from cannulation, the rats were dosed orally with varying doses of the Bt proteins or reference compounds dissolved in either 5% BSA or 5% aqueous tomato extract as a vehicle. Blood samples were collected in herparin treated tubes from the vena porta at -25 hours, +0.05, +0.5, +1.0, +2. +3, +4, +6, +7 and +7.5 hours after dosing (approximately 0.2ml per sample time). The levels of Bt in the blood were analyzed by an ELISA. The dosing solutions were prepared freshly for each administration. Matrix influences were examined by the use of a 10% (w/v) suspension of lyophilized transgenic or non-transgenic tomato tissue in 0.9% saline immediately before use. These suspensions were neutralized with NaOH, clarified by centrifugation (10,000 x g for 5 min.) then diluted 1:1 with a Bt protein stock solution. The vehicles used for the controls were adjusted to have similar protein and buffer compositions. There were nine experiments described in the

accompanying table all of which were dosed with 5 ml of test solution containing from 2.6 to 298mg/kg of Cry9C.

Experiment	Annex 1	Cry9C Bt protein dose (mg/kg)	No. of male rats	ml/animal
1	4	2.6 ± 0.1	6	5.0
2	5	41.9 ± 1.8	4	5.0
3	6	272.0 ± 27.0	9	5.0
4	7	298 ± 6.0	4	5.0
5	8	198 ± 2.9	4	5.0
6	9	234.7 ± 8.1	4	5.0+TM
7	10	229.0 ± 11.6	4	5.0+Bt-TM
8	11	10.6 ± 0.7*	4	5.0
9	12	10.6 ± 0.3*	4	5.0+TM

*Cry1Ab5; TM= tomato matrix San Marzano TL-0001; Bt-TM=Bt-tomato matrix San Marzano RLE15-0001

The validity of the animal model for bioavailability was checked using oral dose of the following substances: ^{14}C-PEG, a marker of paracellular transport at 19.3-20.3 µCi/rat and ^{3}H-propranolol, a marker of transcellular transport at 29.2-30.7 µCi/rat.

Examination of gastro-intestinal tract tissue. At terminal sacrifice, samples were taken from the GI tract for sectioning and histological examination (including immuno-staining). The following tissues were examined: midsegment stomach (between fore and glandular stomach), a duodenal segment (3cm distal from pylorus), a jejunal segment (3cm distal from the ilexura duodenojejunalis 40cm from the pylorus), an ileosegment (3cm proximal from the ileocaecalis transition), a midsegment of the caecum and a colonic segment (3cm distal from the valva ileocaecalis). The gut tissue samples were preserved in Bouin Hollande's 10% sublimate fixative, embedded in paraffin wax, sectioned at 5µm and stained with haematoxylin/eosin. Gut tissue of Lys mutant Cry9C treated rats were also stained immunocytochemically to determine binding of the Cry9C protein.

Gastric and luminal debris was collected from the remaining parts of the GI tract after opening these fragments longitudinally. Aliquots of chyme were immediately diluted 1:1 in PBS (pH8.5) with 1.5mM PMSF and 2.0mM EDTA and stored at -80°C until analysis. The procedure was validated by Bt protein spiking in the range of 0.05 to 0.5 ng/mg luminal debris. Feces were pooled over 24 hours and stored frozen at -80°C until analysis. Aliquots of frozen feces were diluted 1:1 in 50mM TrisHCl (pH7.3) containing 1.0mM PMSF and 2.0mM EDTA, extracted for one hour on ice and the supernatant diluted 1:1 with 10.0mM TrisHCl (pH8.0) containing 1.0mM EDTA, 20% glycerol, 5%

SDS, 2% dithiothreitol and 0.2% bromophenol blue. This solution was heated to 100°C for 5 minutes and stored at -40°C until western blotting.

Blood samples from the labeled PEG and propranolol treatments were examined by liquid scintillation counting after placing 150µl of serum in 3ml of PicoAqua and shaking until clear. Fifty mg samples of feces or chyme were diluted in 0.5ml of water, rehydrated for 0.5 hours, suspended in 3.0ml of Soluene 350, swirled and incubated at 60° overnight. After this incubation, 0.5ml of 2-propanol was added and incubated for 5 minutes at 60°C, then 0.2ml of 30% hydrogen peroxide were added three times and incubated for 2 hours. After cooling to room temperature, 15 ml of Hionic fluor was added to the samples and they were counted.

Enzyme linked immunosorbent assay (ELISA) An indirect double antibody sandwich assay was used to detect levels of the Lys mutant Cry9C in blood. The ELISA was performed according to SOP PGS-9D/1 which was not included. The coating/capture antibody was RÓCry9C followed by a 1:1000 dilution of GÓCry9C as the secondary antibody and a 1:10,000 dilution of RÓGIgG-HRP conjugate as the detection antibody. The sensitivity of the ELISA technique was checked by running spiked samples of blood and chyme in sixfold dilutions from 1 to 100 ngBt protein/ml sample.

SDS-PAGE was performed using a Pharmacia Multifor II horizontal electrophoresis apparatus with ExcelGel homogenous 12.5% SDS gels. The protein samples to be analyzed were prepared by 1:1 dilution with Laemmli sample buffer with dithiothreitol and boiled at 100°C for 5 minutes.

Iso-electric focusing was performed on a Pharmacia Multifor II horizontal electrophoresis apparatus with ready to use Ampholyte buffer strips (pH 3.0-10.0). The samples were prepared according to manufacturer's manual which was not included in the submission.

2D-gel electrophoresis was performed on a Pharmacia Multifor II horizontal electrophoresis apparatus with ready to use Ampholyte buffer strips (pH 3.0-10.0) and ExcelGel homogenous 12.5% SDS gels. The samples were prepared according to manufacturer's manual which was not included in the submission.

Capillary zone electrophoresis was done with a Spectra Phoresis 1000 (Thermo Separation Products) equipped with a fused silica capillary (70 cm x 75 µm, Thermo Separation Products) or a hydrophilic coated capillary (70 cm x 75 µm) CElect P175 (Supelco). The capillary was cleaned by rinsing with 0.1N NaOH at the beginning of the day and allowed to equilibrate with a running buffer rinse for about 2-5 minutes prior to the first sample. The buffer reservoir at the cathode was filled with the same running buffer. The 20nl samples were hydrodynamic (in vacuo) for 5 seconds during injection and introduced at the anode. The voltage across the capillary was 15kV.

Protein band visualization was accomplished for any of the above protein separation techniques by either Coomassie brilliant blue G-250 staining or by silver staining.

Western blotting was performed on the Bt proteins or their proteolytic fragments after they were first separated by SDS-PAGE. The proteins in the SDS-PAGE gels were transferred to either nitrocellulose or Hyperbond-H membranes by semi-dry blotting between two graphite plates. The

membranes were blocked for non-specific binding of antibodies with BSA then probed for the presence of Cry proteins with a 1:1000 dilution of Cry specific rabbit polyclonal antibodies. After rinsing, any bound specific rabbit antibody was detected using a goat anti-rabbit IgG antibody conjugate with alkaline phosphatase. The bound antibody complexes were visualized by the chromogenic substrate BCIP/NBT. Protein recovery and extent of degradation was determined by scanning band intensity in a Scanmaster desk-top flat bed scanner compared to reference bands as analyzed by Diversity One software. For the less intense anti-cry protein bands, ECL methodology (Amersham) was used to boost sensitivity.

Receptor binding assay The *in vitro* binding of the Lys mutant Cry9C protein to intestinal tissues of rats or rhesus monkeys was done by an immunocytological method. Basically deparaffinated, rehydrated sections were incubated with Lugol solution and 5% sodium thiosulphate. These sections were then rinsed and incubated with methanol and 0.6% hydrogen peroxide to inactivate the endogenous peroxidases then the rinsed sections were incubated with a Cry9C solution (50µg/ml) overnight at 4°C. Any bound Cry9C was detected by binding of a rabbit polyclonal anti-Cry9C antibody and visualized with a sheep anti-rabbit antibody conjugate with HRP (1 hour incubation at room temperature). The bound HRP conjugate was visualized by the reaction of DAB and the other cells were stained with heamalin, dehydrated and covered with mounting medium. Gut membranes of *Maduca sexta* and *Ostrina nubilalis* were prepared to provide positive binding controls and specificity of the assay was confirmed by exclusion of the primary or secondary antibody or inclusion of a rabbit antibody to BSA.

Osmotic resistance assay was done according to the protocol described in Noteborn *et al.*, (1995, ACS symposium Series 605, p. 134-147). This reference was not provided.

RESULTS AND DISCUSSION

The cited data from the submission is included in an appendix to this review.

Characterization of test material

All three batches of Lys mutant Cry9C were shown by SDS-PAGE and western blot to be substantially made up of Cry9C protein compared to the 100% standard provided by PGS. Greater than 90% of each preparation had the 68 kDa band as the major component with the majority of the remaining contaminant being the 55 kDa degradation form. Other minor bands were seen but there amounts or molecular weights were not stated nor were the gels used for justifying these claims presented. The capillary zone electrophoresis results indicated similar findings with there being two major peaks, 85-90% being the 68kDa form and 55kDa form being the other peak.

The batches were said to vary from each other and the supplied standard but no attempt was made to qualify the differences and none of these variations were claimed to alter the statement of > 90% Cry9C purity given above. The stated sensitivity of the gel assays was 1-2ng protein/band. The Cry9C protein was soluble to 18.0mg/ml in a buffer above pH 8.0. The ELISA results were used to calculate all the dose amounts even though it was stated that this assay consistently overestimated the protein content compared to conventional protein assays corrected for purity by about 5%.

Stability to *in vitro* digestion and heat

The three batches of Lys mutant Cry9C either from *E.coli* (batches I & II) or *B. thuringiensis* (batch III) were shown to be substantially resistant to degradation after 2 hours by either SGF or SIF. The samples were taken from SGF or SIF incubation solution and loaded at 19.5µg Cry9C/lane on the gels. In SGF there was an initial decrease of 15-25% compared to the Cry9C buffer control after 15 minutes incubation which did not decrease further after 2 hour's total SGF incubation. There was no decrease at all in the Cry9C concentration resulting from SIF incubation. Two silver stained gels were presented for each of the *in vitro* digestions. In one of the SGF gels (figure 3) it is curious to note that no 55kDa form of Cry9C is present in the gel although this is stated as always being a major contaminant.

When the Cry9C samples were subject to heat (90°C) for extended periods the protein appear to be stable as the protein banding pattern in SDS-PAGE gels is unaltered. The presence of a tomato matrix does not appear to affect this heat stability. However, the company has presented an isoelectric focusing gel which purports to show a loss of detectable protein forms after 30 to 60 minutes. They state that this contradictory data is due to the spontaneous aggregation of Cry9C into higher molecular weight forms. None of these higher molecular weight aggregate forms are seen in the SDS-PAGE gels. It is unclear how SDS-PAGE alone would detect heat stability since all samples are routinely heated prior to gel analysis. Probably more appropriate endpoint to measure thermostability would be an insect bioassay which would at least detect degradation from the active, toxic 68kDA to the non-toxic 55kDa form.

Stability to *in vivo* digestion

Nine male Wistar rats were dosed with 83 mg of Cry9C by oral gavage equivalent to a dose of approximately 283±20mg/kg bodyweight. Eight hours after dosing, gastric and luminal debris were collected, separated by SDS-PAGE and the molecular weight and stability were examined by immunoblot (figure 13). While the gel only includes 4 rats, the results indicate that the Cry9C protein is degraded to the 55kDa from over time and that is the major immunoreactive band found in the caecum and colon.

Bioavailability in the rat

Data using radiolabelled PEG and propranolol were used to validate that artificial substances can be tracked in rats and mimic the absorption of food proteins. Also assumptions about bioavailability between a large molecular weight compound (not readily absorbed) and a small model solute (rapidly absorbed as a first order rate process) were valid. The data show that GI passage is about 24 hours and that a total of approximately 30% of the model solute propranolol is bioavailable whereas about 0.03% of the large molecular weight compound PEG is bioavailable. No indication of the amount of the PEG or propranolol administered was given although the radioactivity counts were well described.

The data for bioavailability of Cry9C protein was monitored by the indirect double antibody sandwich ELISA previously described which had a detection limit of 2.0 ng Cry9C/ml in serum with a recovery rate of 85±5%. Previous work with Cry1Ab5 is also presented and that ELISA had a detection limit of 5.0 ng Cry1Ab5/ml with a recovery rate of 55±5%. The animals were dosed with a range of Cry9C from 2.6 to 298 mg/kg bodyweight by gastric intubation. All the dosed animals

appeared and behaved normally, survived to sacrifice and displayed no treatment related adverse effects.

No ELISA positive Cry9C protein or fragments were detected in blood samples taken from rats at the 2.6mg/kg dose. At the higher dose rates, Cry9C ELISA values were positive giving a calculated value of between 5 and 15 ng Cry9C/ml in serum. The absorption rate was not affected by dose amount or presence of a food matrix. The percentage of bioavailable Cry9C was calculated to range from 0.0006% (42mg/kg) to 0.0002% (298mg/kg) of the dose administered.

More importantly, an SDS-PAGE/western blot confirmation of the sera that were ELISA positive for the presence of Cry9C protein showed that only one of the rats had serum with confirmed Cry9C protein. This rat (#548) had a faint Cry9C positive band, all the other sera had no detectable Cry9C reactivities. No interfering compounds were found in rat serum or BSA. The positive signals were were attributed to cross-reaction with Cry9C like components present in both control and Bt-treated animals since there were no significant differences between the 2-D electrophoresis or ECL results. This data indicating cross reaction was not presented.

Immunocytochemical analysis and hemolytic effects

Intestinal tissue from rats, rhesus monkeys and humans was used to examine the binding of Cry9C to the intestinal membranes in tissue sections. A rabbit polyclonal IgG antibody was raised against Cry9C as the detection reagent. A positive Cry9C binding control using sections of brush border epithelial cells from *Ostrinia nubilalis* or *Manduca sexta* was also examined. No positive specific binding was reported for the non-arthropod species tested. However, a high degree of nonspecific antibody binding to the tissues from *Ostrinia nubilalis* or *Manduca sexta* even in the absence of Cry9C was reported. This background binding was difficult to reduce but the best positive reaction was obtained with *Ostrinia nubilalis* tissues using 3% BSA as a blocking agent and increasing the Cry9C incubations to 15 minutes.

Examination of the GI tissue samples from the nine rats in the high dose Cry9C group revealed no Cry9C binding to any tissue of the GI tract. The villi and individual enterocytes lining the crypts of the small and large intestines were normal compared to these same tissue sections in the untreated controls.

Human red blood cells were examined for any hemolytic effects after incubation with Cry9C by monitoring for osmotic fragility spectroscopically. No hemolysis was indicated by the assay.

No appendix 5 is included in the submission although many of the techniques refer to it.

APPENDIX to BIOAVAILABILITY

ANNEX 1 - Table 2

LYS MUTANT CRY9C ORAL STABILITY TO DIGESTION in vitro
RIKILT-DLO
Study: Digestibility *in vitro* - Simulated Intestinal Fluid (SIF)

Table 2 Stability as percentage (%) of parent band (native protein)[1]

Time (min)		Protein				
		Cry9C	Cry1Ab5	CryIIIb	NPTII	PAT
0	Mean	100	100	100		0
2	Mean	100	100	100		10
	SD					7
	n	4	4	4		4
5	Mean	100	100	100		3
	SD					1
	n	4	4	4		4
15	Mean	100	100	1010		0
	SD					
	n	4	4	4		4
30	Mean	100	100	100		0
	SD					
	n	4	4	4		4
60	Mean	100	100	100		0
	SD					
	n	4	4	4		4
120	Mean	100	100	100		0
	SD					
	n	4	4	4		4

Statistics:
ANOVA: * $P < 0.05$ ** $P < 0.01$
[1]: western blots measured by scanning densitometer
The absence of a percentage indicates that the process specified was not recorded at that time point.

ANNEX 1 - Table 1

LYS MUTANT CRY9C ORAL STABILITY TO DIGESTION in vitro
RIKILT-DLO
Study: Digestibility *in vitro* - Simulated Gastric Fluid (SGF)

Table 1 Stability as percentage (%) of parent band (native protein)[1]

Time (min)		Protein				
		Cry9C	Cry1Ab5	CryIIIb	NPTII	PAT
0	Mean	100	100	100	0	0
2	Mean	96	41	13	9	50
	SD	4	9	7	3	10
	n	4	4	4	4	4
5	Mean	86		0	0	0
	SD	3				
	n	4		4	4	4
15	Mean	82	21	0	0	0
	SD	5	5			
	n	4	4	4	4	4
30	Mean	78	21	0	0	0
	SD	6	8			
	n	4	4	4	4	4
60	Mean	77	11	0	0	0
	SD	3	6			
	n	4	4	4	4	4
120	Mean	78	9	0	0	0
	SD	5	4			
	n	4	4	4	4	4

Statistics:
ANOVA: * $P < 0.05$ ** $P < 0.01$
[1]: western blots measured by scanning densitometer
The absence of a percentage indicates that the process specified was not recorded at that time point.

24B

ANNEX 1 - Table 4

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT
RIKILT-DLO
Study: Wistar (6 males) - bleeding from portal vein canula
Dose: 1.0 mg Lys mutant Cry9C/rat/5.0 ml of 0.9% NaCl (pH 7.2) containing 5% (w/v) BSA

Table 4 Plasma levels of Cry9C measured by DS-ELISA

Time (hrs)		Concentration (ng/ml of plasma) in the various animals					
		521 (418 g)	522 (391 g)	523 (368 g)	524 (407 g)	525 (419 g)	528 (357 g)
0	Mean 0 SD	0	0	0	0	0	0
0.05	Mean SD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
0.5	Mean SD	n.d.	n.d.	n.d.	n.d.		n.d.
1.0	Mean SD		n.d.	n.d.	n.d.	n.d.	n.d.
2.0	Mean SD	n.d.	n.d.	n.d.			n.d.
3.0	Mean SD.		n.d.	n.d.	n.d.	0.81	n.d.
4.0	Mean SD	n.d.	n.d.	0.8			n.d.
6.0	Mean SD		n.d.	0.88	n.d.		n.d.
7.0	Mean SD	n.d.	n.d.	1.14	n.d.	1.38	n.d.
7.5	Mean SD						
	Dose (mg/kg) Mean 2.57 SD 0.14	2.4	2.6	2.7	2.5	2.4	2.8

Statistics:
ANOVA: * $P < 0.05$ ** $P < 0.01$
n.d.: not detectable
The absence of a concentration indicates that the blood sample specified was not taken.

24C

ANNEX 1 - Table 5

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT
RIKILT-DLO
Study: Wistar (4 males) - bleeding from portal vein canula
Dose: 13 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) BSA

Table 5 Plasma levels of Cry9C measured by DS-ELISA

Time (hrs)			Concentration (ng/ml of plasma) in the various animals			
			569 (331 g)	573 (309 g)	575 (317 g)	576 (289 g)
0	Mean	0	0	0	0	0
	SD					
0.05	Mean	3.2	3.48	2.32	5.07	1.81
	SD	1.4				
0.5	Mean	5.2		3.94	5.61	6.03
	SD	0.9				
1.0	Mean	5.8		4.3	7.24	
	SD	1.5				
2.0	Mean	6.7	7.87	4.23	5.61	9.1
	SD	2.2				
3.0	Mean	3.9		2.93	4.78	
	SD	0.9				
4.0	Mean	4.7	4.56	0.85	4.65	8.82
	SD	2.8				
6.0	Mean					
	SD					
7.0	Mean	5.2	3.47	1.31	3.74	12.27
	SD	4.2				
7.5	Mean	8.0	3.67	1.74	2.03	24.39
	SD	9.5				
	Dose (mg/kg)		39.3	42.1	41.0	45.0
	Mean	41.9				
	SD	1.8				

Statistics:
ANOVA: * $P < 0.05$ ** $P < 0.01$
The absence of a concentration indicates that the blood sample specified was not taken.

ANNEX 1 - Table 6

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT
RIKILT-DLO
Study: Wistar (9 males) - bleeding from portal vein canula
Dose: 83 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) BSA

Table 6 Plasma levels of Cry9C measured by DS-ELISA

Time (hrs)			Concentration (ng/ml of plasma) in the various animals								
			547 (295 g)	548 (287 g)	549 (275 g)	551 (262 g)	556 (352 g)	558 (342 g)	561 (340 g)	562 (327 g)	550 (293 g)
0	Mean		0	0	0	0	0	0	0	0	0
0.05	Mean	0.8	0.88	0	0	0	0.8	0	0	0.9	0
	SD										
0.5	Mean	16.8	23.7	74.6	21.2	16.4	1.2	13.5	0	0.8	0
	SD	22.3									
1.0	Mean	6.9	2.1	6.5		22.0	3.5	17.7	0.7	1.2	1.6
	SD	7.7									
2.0	Mean	10.3	4.7	3.3		27.7	7.4	20.9	6.8	7.0	4.8
	SD	8.3									
3.0	Mean	16.2	4.0	3.7		29.7	7.6	20.4	31.2	27.8	5.0
	SD	11.5									
4.0	Mean	15.5	12.5	2.4	12.7	31.4	15.6	20.2		23.4	5.4
	SD	8.8									
6.0	Mean	16.3	3.4	1.2		23.7	14.8	34.6		33.3	3.4
	SD	13.3									
7.0	Mean	14.9	11.7	6.7		37.1	12.7	21.0	5.6	12.4	12.3
	SD	9.4									
7.5	Mean	10.3	3.73	4.3	3.4	28.6	10.0	17.2	6.1	10.4	9.2
	SD	7.7									
	Dose (mg/kg)		281.4	289.2	301.8	316.8	235.8	242.7	253.8	244.1	283.3
	Mean	272.1									
	SD	27.3									

Statistics: ANOVA * $P < 0.05$ ** $P < 0.01$
Rat no. 555 (334 g) and 560 (342 g) were dosed with vehicle only and served as DS-ELISA controls.
The absence of a concentration indicates that the blood sample specified was not taken.

ANNEX 1 - Table 7

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT
RIKILT-DLO
Study: Wistar (4 males) - bleeding from portal vein canula
Dose: 91 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) BSA

Table 7 Plasma levels of Cry9C measured by DS-ELISA

Time (hrs)			Concentration (ng/ml of plasma) in the various animals			
			564 (303 g)	565 (310 g)	568 (310 g)	574 (298 g)
0	Mean	0	0	0	0	0
	SD					
0.05	Mean	1.60	1.84	1.55	1.14	1.85
	SD	0.3				
0.5	Mean	5.49	5.92	6.55	4.80	6.03
	SD	0.6				
1.0	Mean	9.97	7.17	19.83	6.99	5.03
	SD	6.5				
2.0	Mean	7.51	8.08	13.83	6.01	5.1
	SD	2.1				
3.0	Mean	6.32	7.44	15.07	4.39	2.2
	SD	2.7				
4.0	Mean	6.18	6.92	15.70	3.75	3.82
	SD	5.8				
6.0	Mean	4.01	2.84	10.28	2.93	2.27
	SD	1.6				
7.0	Mean	2.35	1.61	8.31	0.89	1.41
	SD	2.1				
7.5	Mean	6.6	3.02	9.4	8.5	2.0
	SD	2.7				
	Dose (mg/kg)		300.3	293.5	293.5	305.4
	Mean	298.2				
	SD	5.7				

Statistics: ANOVA * P< 0.05 ** P< 0.01
The absence of a concentration indicates that the blood sample specified was not taken.

24F
037

ANNEX 1 - Table 8

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT
RIKILT-DLO
Study: Wistar (4 males) - bleeding from portal vein canula
Dose: 60 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) BSA

Table 8 Plasma levels of Cry9C measured by DS-ELISA

Time (hrs)			Concentration (ng/ml of plasma) in the various animals			
			530 (301 g)	531 (296 g)	532 (306 g)	534 (307 g)
0	Mean	0	0	0	0	0
	SD					
0.05	Mean	1.6	1.69	1.12	2.32	1.23
	SD	0.4				
0.5	Mean	5.8	6.82	6.45	4.98	4.78
	SD	0.9				
1.0	Mean	7.2	6.91	7.76	9.09	5.08
	SD	1.5				
2.0	Mean	8.7	10.77	10.02	7.56	6.34
	SD	1.8				
3.0	Mean	7.9	8.9	9.72	8.06	5.1
	SD	1.7				
4.0	Mean	5.5	6.45	7.13	5.91	2.58
	SD	1.8				
6.0	Mean					
	SD					
7.0	Mean					
	SD					
7.5	Mean	2.1	1.23	3.23	2.92	1.1
	SD	1.0				
	Dose (mg/kg)		199.3	202.7	196.1	195.4
	Mean	198.4				
	SD	2.9				

Statistics:
ANOVA: * $P < 0.05$ ** $P < 0.01$
The absence of a concentration indicates that the blood sample specified was not taken.

ANNEX 1 - Table 9

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT
RIKILT-DLO
Study: Wistar (4 males) - bleeding from portal vein canula
Dose: 71.6 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) tomato matrix (variety: TL-0001)

Table 9 Plasma levels of Cry9C measured by DS-ELISA

Time (hrs)			Concentration (ng/ml of plasma) in the various animals			
			563 (310 g)	567 (316 g)	570 (311 g)	577 (285 g)
0	Mean	0	0	0	0	0
	SD					
0.05	Mean	2.5	1.46	0.92	5.02	2.69
	SD	1.6				
0.5	Mean	6.2	5.56	6.12	6.96	6.23
	SD	0.5				
1.0	Mean	8.7	5.19	9.85	13.95	5.91
	SD	3.5				
2.0	Mean	10.9	7.07	14.56	8.14	9.1
	SD	3.3				
3.0	Mean	8.9	6.38	10.72	8.43	13.77
	SD	1.7				
4.0	Mean	6.0	3.69	7.45	5.62	10.04
	SD	1.5				
6.0	Mean					
	SD					
7.0	Mean					
	SD					
7.5	Mean	8.5	3.48	14.29	9.65	7.13
	SD	4.0				
	Dose (mg/kg)		230.9	226.5	229.5	251.2
	Mean	234.7				
	SD	8.1				

Statistics:
ANOVA: * $P < 0.05$ ** $P < 0.01$
The absence of a concentration indicates that the blood sample specified was not taken.

24 H
033

ANNEX 1 - Table 10

LYS MUTANT CRY9C ORAL BIOAVAILABILITY RAT
RIKILT-DLO
Study: Wistar (4 males) - bleeding from portal vein canula
Dose: 69.6 mg Lys mutant Cry9C/rat/5.0 ml of 20 mM TrisHCl/0.5 mM NaEDTA (pH 8.6) containing 5% (w/v) Bt-tomato matrix (variety: RLE15-0001)

Table 10 Plasma levels of Cry9C measured by DS-ELISA

Time (hrs)			Concentration (ng/ml of plasma) in the various animals			
			566 (311 g)	571 (276 g)	572 (316 g)	578 (317 g)
0	Mean	0	0	0	0	0
	SD					
0.05	Mean	1.8	1.00	2.15	2.85	1.03
	SD	0.8				
0.5	Mean	6.2	4.62	9.08	3.55	7.62
	SD	2.2				
1.0	Mean	8.6	7.23	11.72	8.3	7.3
	SD	1.8				
2.0	Mean	7.1	8.20	8.64	6.7	5.0
	SD	1.4				
3.0	Mean	6.6	10.73	6.6	4.8	4.4
	SD	2.5				
4.0	Mean	4.0	6.93	4.2	1.9	3.1
	SD	1.9				
6.0	Mean					
	SD					
7.0	Mean	5.3	7.4	7.3	2.4	4.1
	SD	2.1				
7.5	Mean	7.9	9.5	14.6	2.1	5.2
	SD	4.7				
	Dose (mg/kg)		223.8	252.2	220.3	219.6
	Mean	229.0				
	SD	11.6				

Statistics:
ANOVA: * $P < 0.05$ ** $P < 0.01$
The absence of a concentration indicates that the blood sample specified was not taken.

ANNEX 1 - Table 11

CRY1Ab5 ORAL BIOAVAILABILITY RAT
RIKILT-DLO
Study: Wistar (4 males) - bleeding from portal vein canula
Dose: 3.5 mg Cry1Ab5/rat/5.0 ml of 50 mM Na_2CO_3/0.2 mM NaCl (pH 7.2) containing 5% (w/v) BSA

Table 11 Plasma levels of Cry1Ab5 measured by ELISA

Time (hrs)		Concentration (ng/ml of plasma) in the various animals			
		510 (300 g)	514 (332 g)	516 (342 g)	517 (356 g)
0	Mean 0 SD	0	0	0	0
0.05	Mean SD	n.d.	n.d.	n.d.	n.d.
0.5	Mean SD	n.d.	n.d.	n.d.	n.d.
1.0	Mean SD	n.d.	n.d.	n.d.	n.d.
2.0	Mean SD	n.d.	n.d.	n.d.	n.d.
3.0	Mean SD	n.d.	n.d.	n.d.	n.d.
4.0	Mean SD	n.d.	n.d.	n.d.	n.d.
6.0	Mean SD	n.d.			
7.0	Mean SD	n.d.			
24	Mean SD	n.d.	n.d.	n.d.	n.d.
	Dose (mg/kg) Mean 10.6 SD 0.7	11.7	10.5	10.2	9.8

Statistics: ANOVA $P < 0.05$ ** $P < 0.01$
n.d.: not detectable
The absence of a concentration indicates that the blood sample specified was not taken.

24 J
041

ANNEX 1 - Table 12

CRY1Ab5 ORAL BIOAVAILABILITY RAT
RIKILT-DLO
Study: Wistar (4 males) - bleeding from portal vein canula
Dose: 3.5 mg Cry1Ab5/rat/5.0 ml of 50 mM Na_2CO_3/0.2 mM NaCl (pH 7.2) containing 5% (w/v) tomato matrix (variety: TL-0001)

Table 12 Plasma levels of Cry1Ab5 measured by ELISA

Time (hrs)		Concentration (ng/ml of plasma) in the various animals			
		511 (322 g)	515 (342 g)	518 (319 g)	519 (341 g)
0	Mean 0 SD	0	0	0	0
0.05	Mean SD	n.d.	n.d.	n.d.	n.d.
0.5	Mean SD	n.d.	n.d.	n.d.	n.d.
1.0	Mean SD	n.d.	n.d.	n.d.	n.d.
2.0	Mean SD	n.d.	n.d.	n.d.	n.d.
3.0	Mean SD	n.d.	n.d.	n.d.	n.d.
4.0	Mean SD	n.d.	n.d.	n.d.	n.d.
6.0	Mean SD	n.d.	n.d.	n.d.	n.d.
7.0	Mean SD				
24	Mean SD	n.d.	n.d.	n.d.	n.d.
	Dose (mg/kg) Mean 10.6 SD 0.3	10.9	10.2	11.0	10.3

Statistics: ANOVA * $P < 0.05$ ** $P < 0.01$
n.d.: not detectable
The absence of a concentration indicates that the blood sample specified was not taken.

FIGURE 1

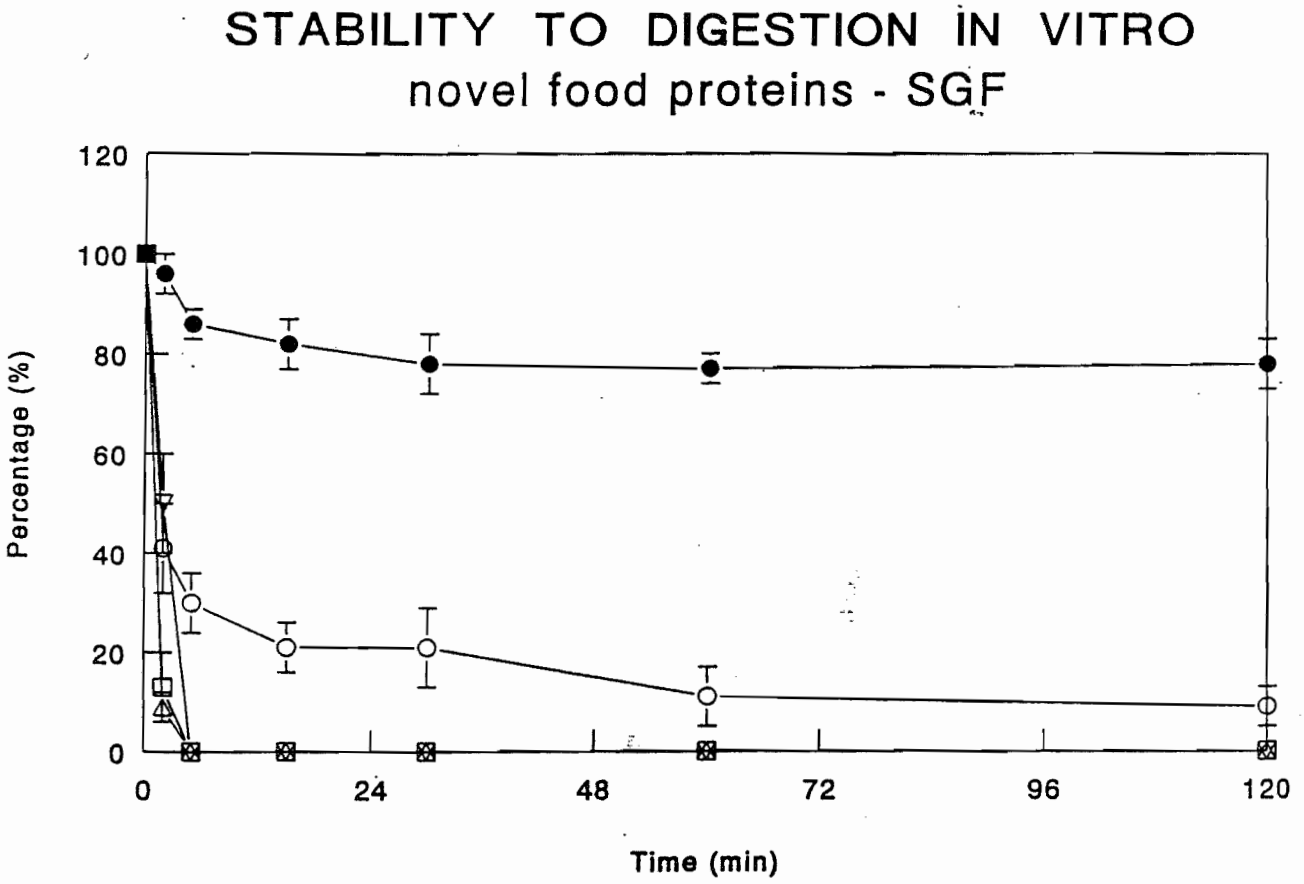


Figure 1.
Representative graph showing the stability of the Lys mutant Cry9C protein to digestion in vitro under simulated human gastric conditions (SGF). The rate of stability of the Cry9C protein was analyzed by scanning of the 1-D lanes with a Desk-Top flat bed densitometer and estimated by integrating the optical densities of matched Cry9C protein bands in the different 1-D lanes (i.e. SDS-PAGE and Western blots) based on a calibration curve and relative (%) to the density of unincubated gastric buffer (pH 2.0) without pepsin but with the Lys mutant Cry9C protein. The rate of stability of the Lys mutant Cry9C protein (closed circles) was also compared with that of the Cry1Ab5 protein (open circles), the CryIIIb protein (open squares), the NPTII enzyme (open triangles) and the PAT enzyme (open reversed triangles). Data represent mean values (± SD) of sixfold measurements per time point.

FIGURE 2

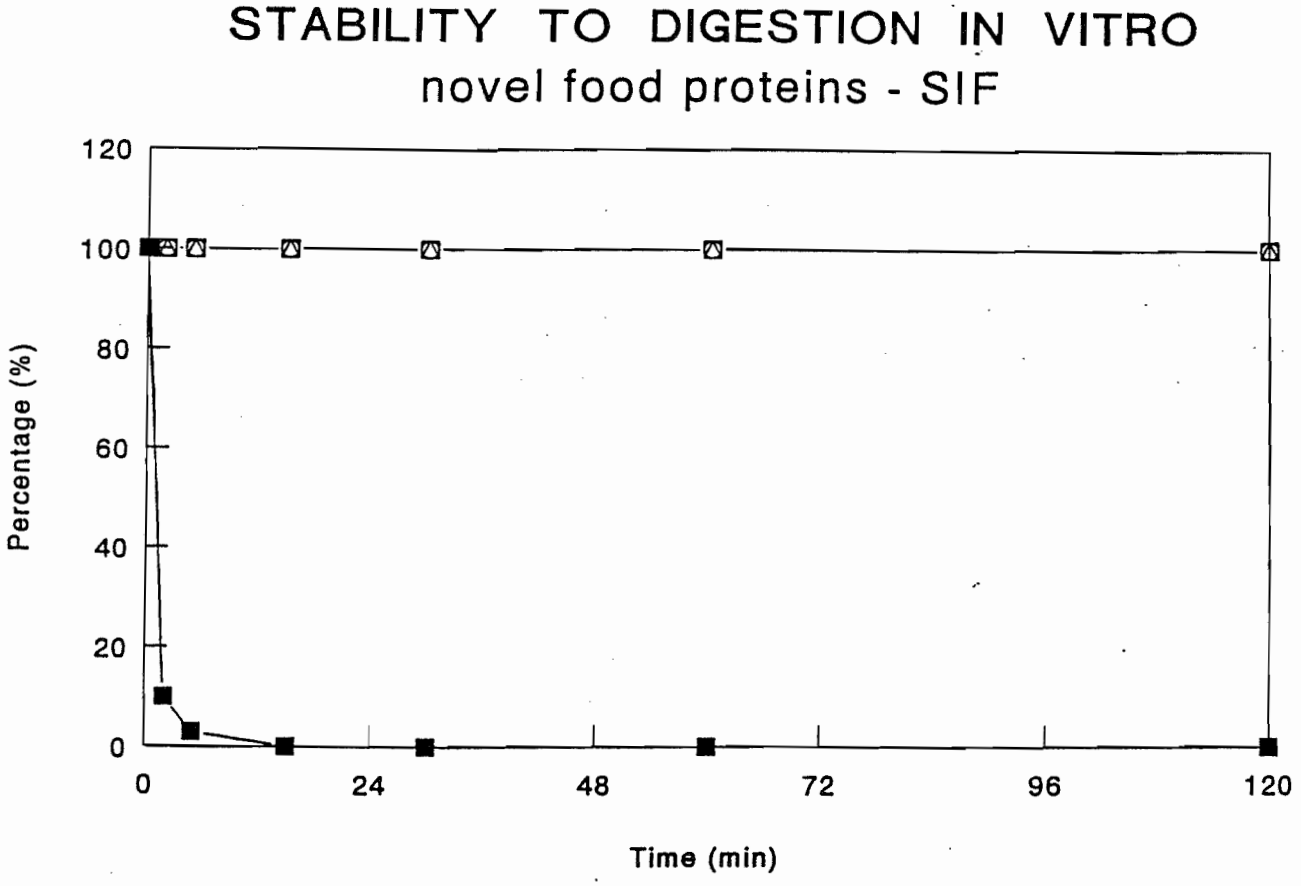


Figure 2.

Representative graph showing the stability of the Lys mutant Cry9C protein to digestion in vitro under simulated human intestinal conditions (SIF). The rate of stability of the Cry9C protein was analyzed by scanning of the 1-D lanes with a Desk-Top flat bed densitometer and estimated by integrating the optical densities of matched Cry9C protein bands in the different 1-D lanes (i.e. SDS-PAGE and Western blots) based on a calibration curve and relative (%) to the density of unincubated intestinal buffer without pancreatin but with the Lys mutant Cry9C protein. The rate of stability of the Lys mutant Cry9C protein (open triangles) was compared with that of the Cry1Ab5 protein (open circles), the CryIIIb protein (open squares) and the PAT enzyme (closed squares). Data represent mean values (± SD) of sixfold measurements per time point.

24 M
048

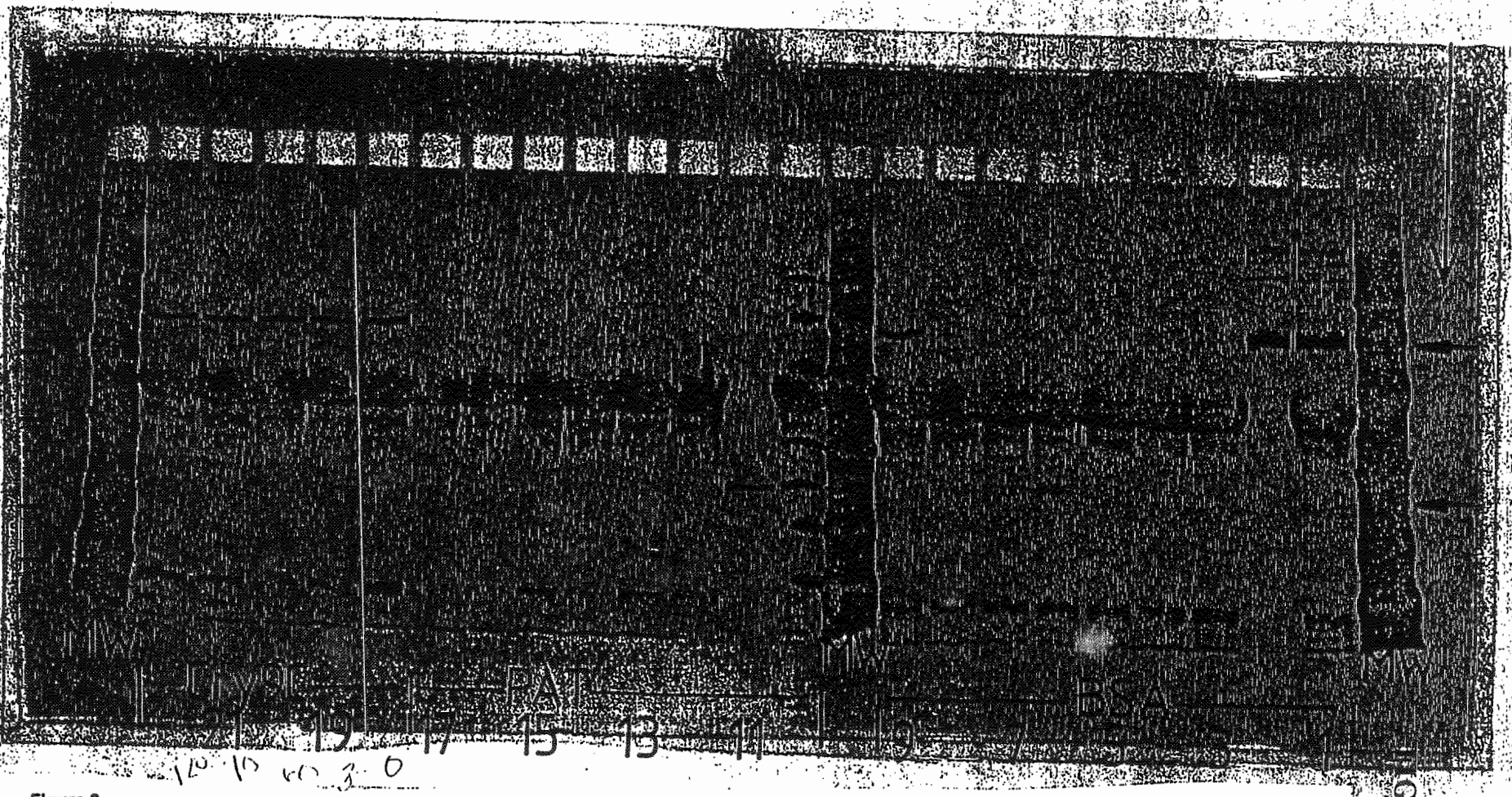


Figure 3.

Profile of the degradation of Lys mutant Cry9C protein observed in simulated gastric fluid as analyzed by SDS-PAGE gel electrophoresis (SDS-PAGE, gel nr. 9611211) and stained with silver. Samples: MW, LMW marker proteins (phosphorylase B, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; Ó-lactalbumin, 14.1 kDa); lanes 18 to 22, Cry9C protein (19.5 µg/lane) taken at 0 min (lane 18), at 30 min (lane 19), at 60 min (lane 20), at 90 min (lane 21) and at 120 min (lane 22) after incubation at 37°C (pH 2) in simulated gastric fluid containing pepsin (SGF); lanes 10 to 17, PAT enzyme (3 µg/lane) taken at 0, 10, 30, 60, 90 and 120 min (lane 12 to 17, respectively) after incubation in SGF, lane 10 quenched unincubated simulated gastric fluid with pepsin and PAT and lane 11, quenched unincubated simulated gastric fluid with PAT and without pepsin; lanes 1-9, BSA (7.5 µg/lane) taken at 0, 10, 30, 60, 90 and 120 min (lane 3 to 8, respectively) after incubation in SGF, lane 1 and 9 quenched (un)incubated simulated gastric fluid with pepsin and BSA and lane 2, quenched unincubated simulated gastric fluid with BSA and without pepsin.

FIGURE 3

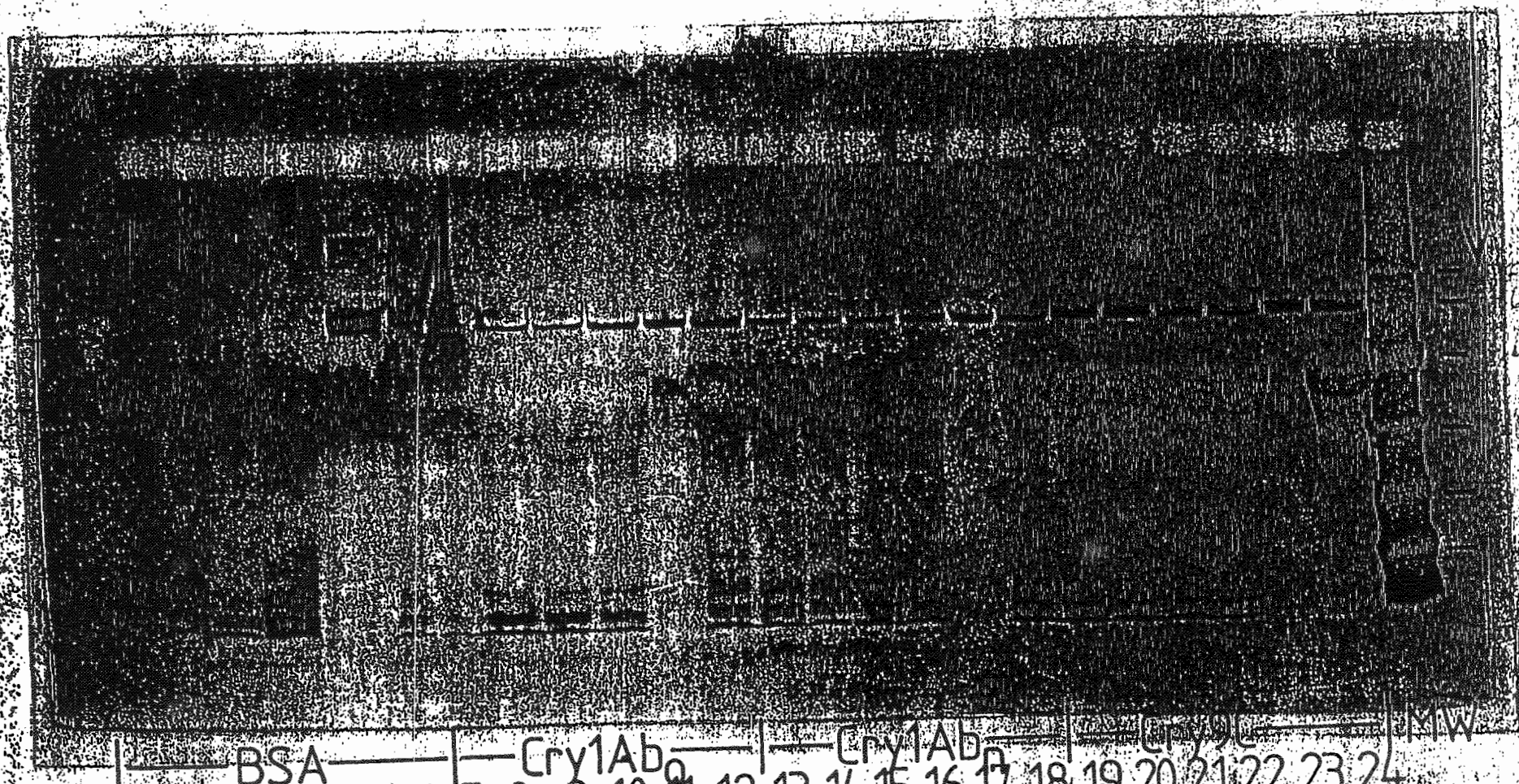


FIGURE 4

Figure 4.
Profile of the degradation of Lys mutant Cry9C protein observed in simulated gastric fluid as analyzed by SDS-PAGE gel electrophoresis (SDS-PAGE, gel nr. 961210) and stained with silver. Samples: MW, LMW marker proteins (phosphorylase B, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; Ò-lactalbumin, 14.1 kDa); lanes 22 to 19, Cry9C protein (19.5 µg/lane) taken at 0 min (lane 22), at 30 min (lane 21), at 60 min (lane 20) and at 120 min (lane 19) after incubation at 37°C (pH 2) in simulated gastric fluid containing pepsin (SGF), lane 24 quenched unincubated simulated gastric fluid with pepsin and Cry9C and lane 23 quenched unincubated simulated gastric fluid with Cry9C and without pepsin; lanes 16 to 13, Cry1Ab5 (batch new, 22.3 µg/lane) taken at 0, 30, 60 and 120 min (lane 16 to 13, respectively) after incubation in SGF, lane 18 quenched unincubated simulated gastric fluid with pepsin and Cry1Ab5 and lane 17 quenched unincubated simulated gastric fluid with Cry1Ab5 and without pepsin; lanes 10 to 7, Cry1Ab5 (batch old, 5.3 µg/lane) taken at 0, 30, 60 and 120 min (lane 10 to 7, respectively) after incubation in SGF, lane 12 quenched unincubated simulated gastric fluid with pepsin and Cry1Ab5 and lane 11 quenched unincubated simulated gastric fluid with Cry1Ab5 and without pepsin; lane 4 to 1, BSA (7.5 µg/lane) taken at 0, 1, 5 and 10 min after incubation in SGF, lane 6 and 5 quenched (un)incubated simulated gastric fluid with pepsin and BSA.

FIGURE 5

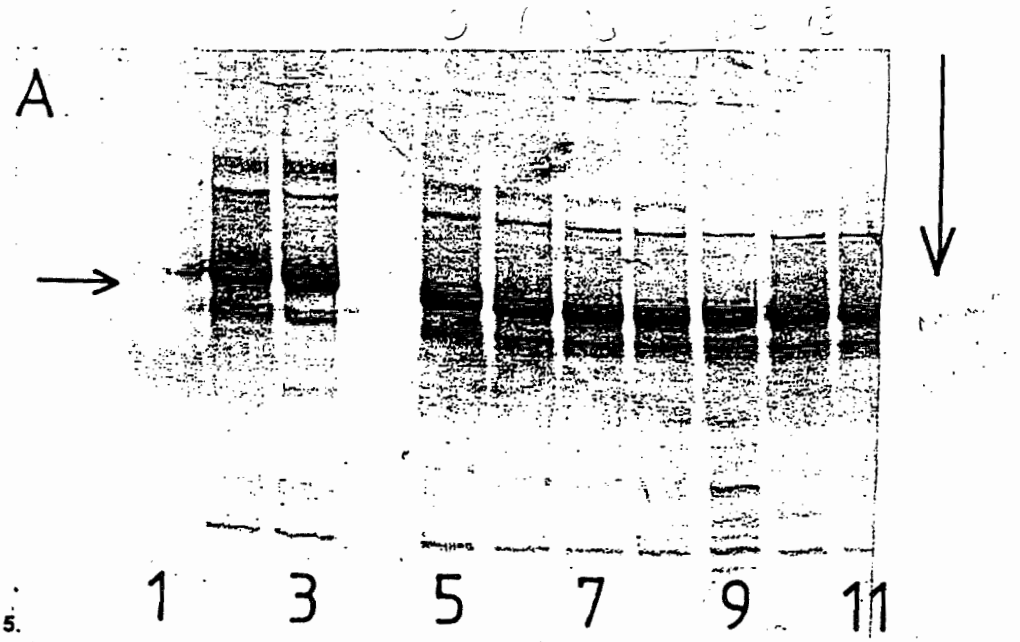


Figure 5.
Profile of the degradation of Lys mutant Cry9C protein observed in simulated gastric (panel A) and intestinal (panel B) fluid as analyzed by western blotting (blot, gel nr. 961030 1/2). Figure 5A: lane 1-2, native Cry9C (6.5 µg/lane), lane lane 3, sample of Cry9C (6.5 µg/lane) taken at 120 min after incubation in SGF with tomato fruit matrix (10% w/v) at 37°C (pH 2.0), lane 4 quenched simulated gastric fluid with pepsin only, lanes 5-11, samples of Cry9C protein taken at 0 min, at 1 min, at 30 min, at 60 min, at 120 min and at 180 min after incubation at 37°C (pH 2.0) in simulated gastric fluid containing pepsin.

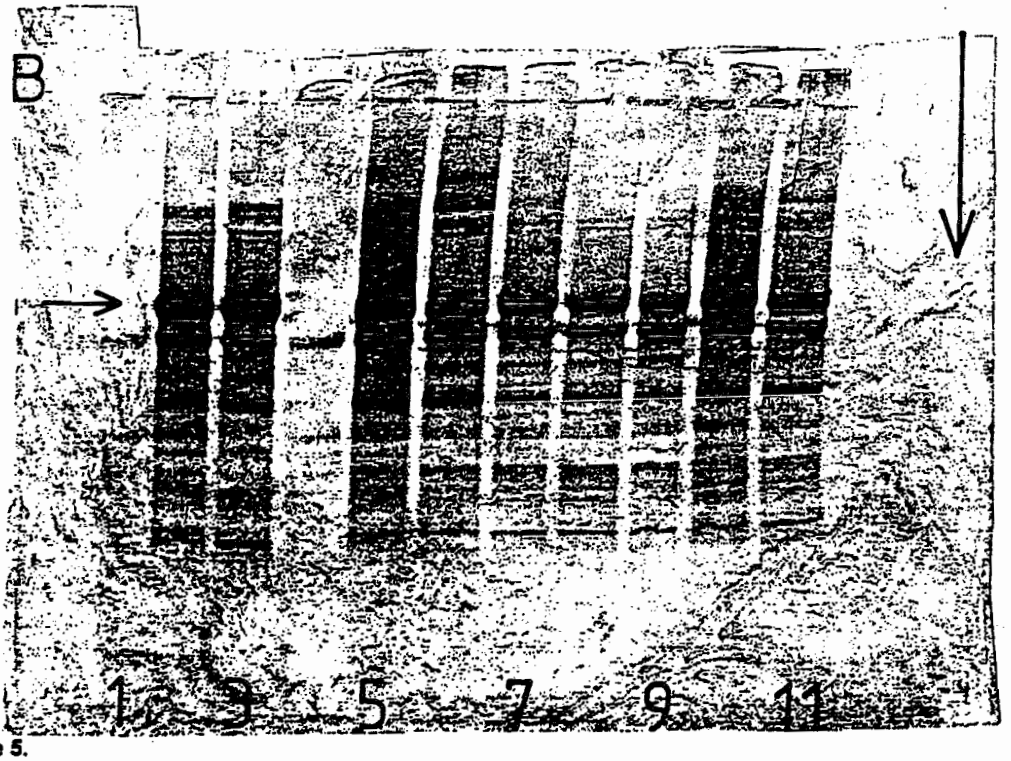


Figure 5.
Profile of the degradation of Lys mutant Cry9C protein observed in simulated gastric (panel A) and intestinal (panel B) fluid as analyzed by western blotting (blot, gel nr. 961030 1/2). Figure 5B: lane 1, LMW markers, lane 2, unincubated simulated intestinal fluid with pancreatin and Cry9C, lane 3, sample of Cry9C taken at 120 min after incubation in SIF with tomato fruit matrix (10% w/v) at 37°C (pH 8.0), lane 4, quenched simulated intestinal fluid with pancreatin only, lanes 5-11, samples of Cry9C protein taken at 0 min, at 1 min, at 30 min, at 60 min, at 120 min and at 180 min after incubation at 37°C (pH 2.0) in simulated intestinal fluid containing pancreatin.

FIGURE 6

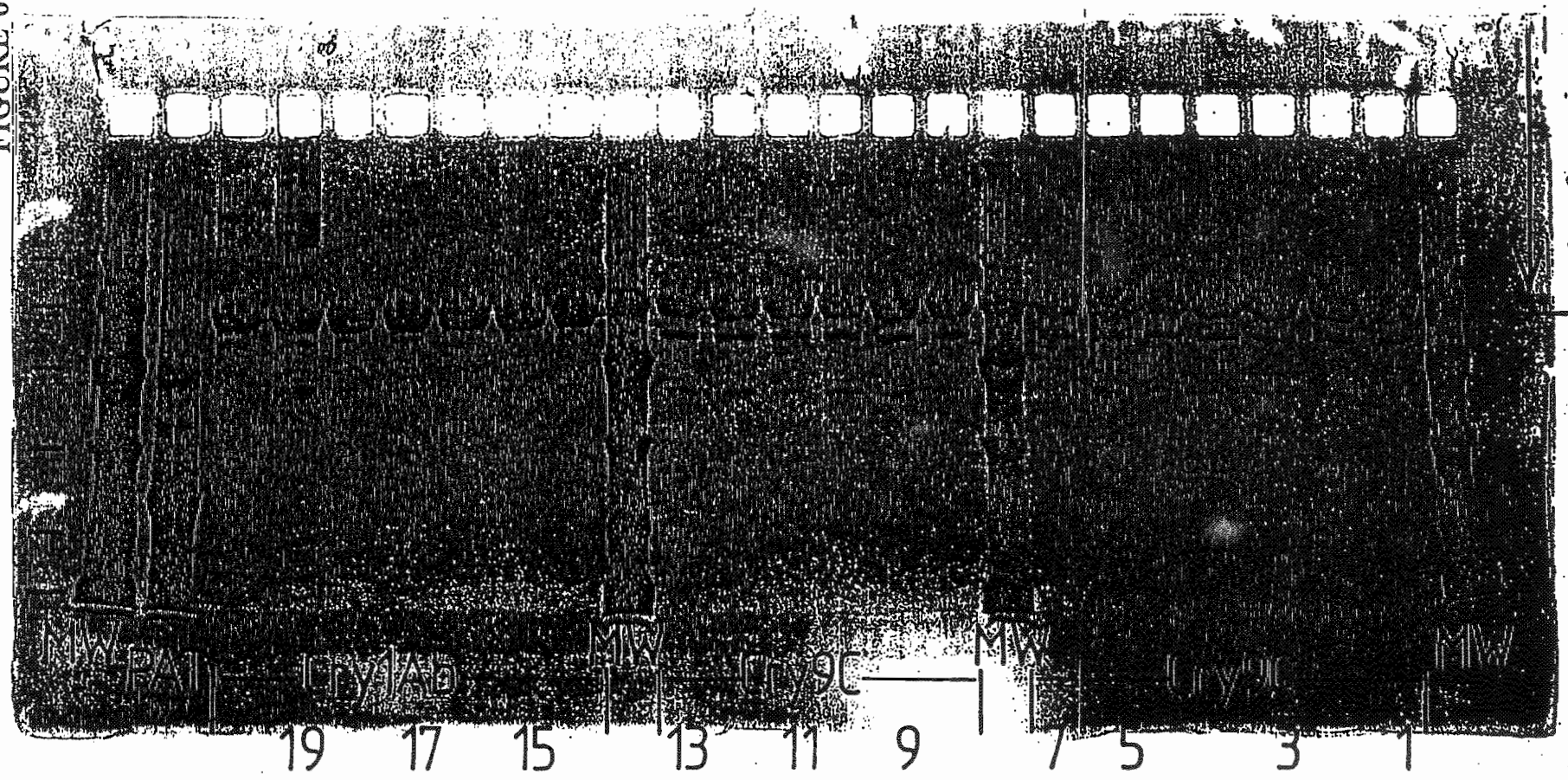


Figure 6.
Profile of the thermostability of Lys mutant Cry9C protein observed in simulated processing conditions as analyzed by SDS-PAGE gel electrophoresis (SDS-PAGE, gel nr. 9606252) and stained with silver. Samples: MW, LMW marker proteins (phosphorylase B, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; Ó-lactalbumin, 14.1 kDa); lanes 1 to 7, Cry9C protein (batch I, 4.4 µg/lane) taken at 0 min (lane 1), at 5 min (lane 2), at 15 min (lane 3), at 30 min (lane 4), at 60 min (lane 5), at 140 min (lane 6) and at 210 min (lane 7) after incubation at 90°C (pH 7.5); lanes 8 to 13, Cry9C protein (batch II, 4.5 µg/lane) taken at 0 min (lane 8), at 5 min (lane 9), at 15 min (lane 10), at 30 min (lane 11), at 60 min (lane 12) and at 210 min (lane 13) after incubation at 90°C (pH 7.5); lanes 14 to 20, Cry1Ab5 protein (3.5 µg/lane) taken at 0 min (lane 14), at 5 min (lane 15), at 15 min (lane 16), at 30 min (lane 17), at 60 min (lane 18), at 140 min (lane 19) and at 210 min (lane 20) after incubation at 90°C (pH 7.5); lane 21, PAT enzyme (4.5 µg/lane) 210 min after incubation at 90°C (pH 7.5)

FIGURE 7

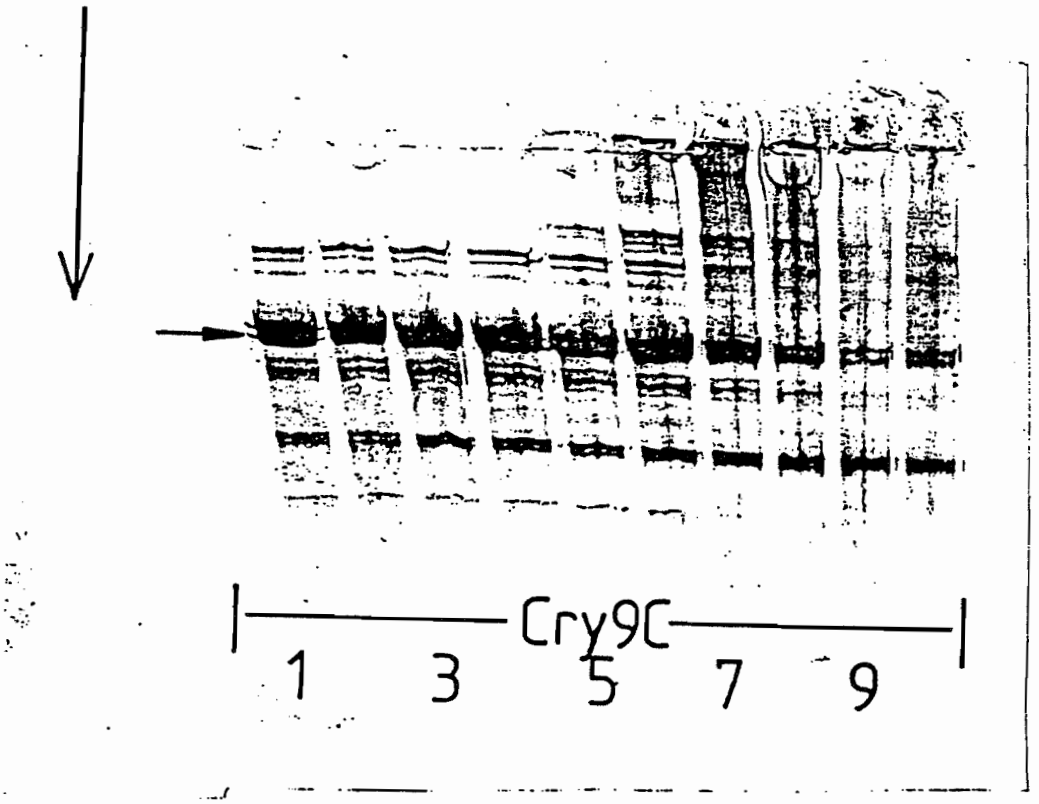


Figure 7.
Profile of the thermostability of Lys mutant Cry9C protein observed in simulated processing conditions as analyzed by western blotting (blot, gel nr. 960827). Samples: lanes 1 to 3, Cry9C protein (batch I, 4.4 µg/lane) taken at 0 min (lane 1), at 60 min (lane 2) and at 210 min (lane 3) after incubation at 20°C (pH 7.5); lanes 4 to 10, Cry9C protein (batch I, 4.4 µg/lane) taken at 0 min (lane 4), at 5 min (lane 5), at 15 min (lane 6), at 30 min (lane 7), at 60 min (lane 8), at 120 min (lane 9) and at 210 min (lane 10) after incubation at 90°C (pH 7.5).

FIGURE 8

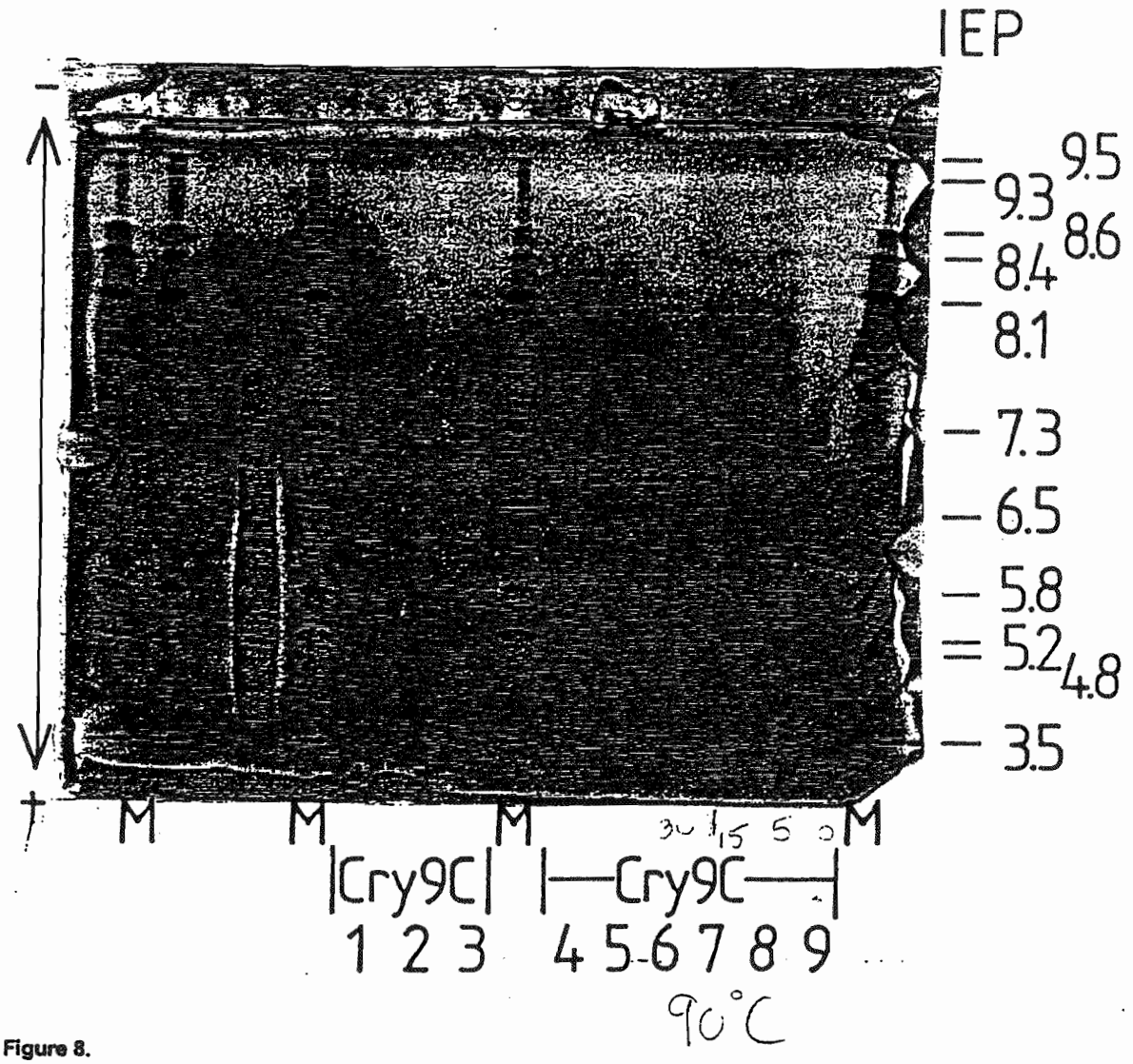


Figure 8.
Profile of the thermostability of Lys mutant Cry9C protein observed in simulated processing conditions as analyzed by iso-electric focusing electrophoresis (pH 3.0 - 10.0, gel nr. 960820). Samples: M, IEP markers; lanes 1 to 3, Cry9C protein (batch I, 4.4 µg/lane) taken at 0 min (lane 1), at 60 min (lane 2) and at 120 min (lane 3) after incubation at 20°C (pH 7.5); lanes 9 to 4, Cry9C protein (batch I, 4.4 µg/lane) taken at 0 min (lane 9), at 5 min (lane 8), at 15 min (lane 7), at 30-min (lane 6), at 60 min (lane 5) and at 120 min (lane 4) after incubation at 90°C (pH 7.5).

FIGURE 9

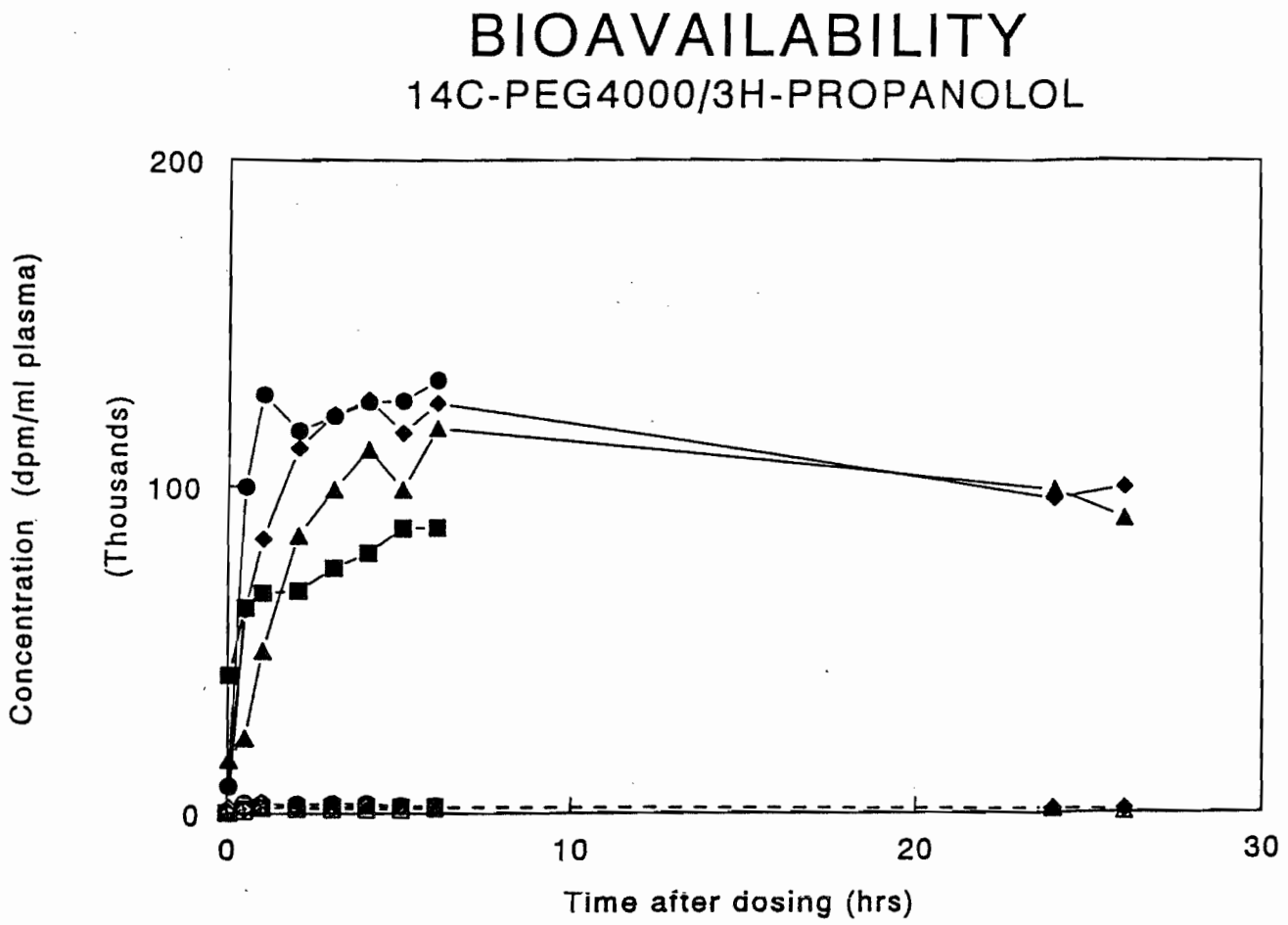


Figure 9.
Uptake of [^{3}H]propanolol and [^{14}C]polyethyleneglycol versus time profiles after oral administration to portal vein canulated rats. The data represent values from two experiments as described in Annex 1 - Table 3. Key: black symbols values of [^{3}H]propanolol (i.e. circles, rat no. 540; squares, rat no. 542; triangles, rat no. 543; diamonds, rat no. 545) and gray symbols values of [^{14}C]polyethyleneglycol (i.e. circles, rat no. 540; squares, rat no. 542; triangles, rat no. 543; diamonds, rat no. 545).

24 T
055

FIGURE 10

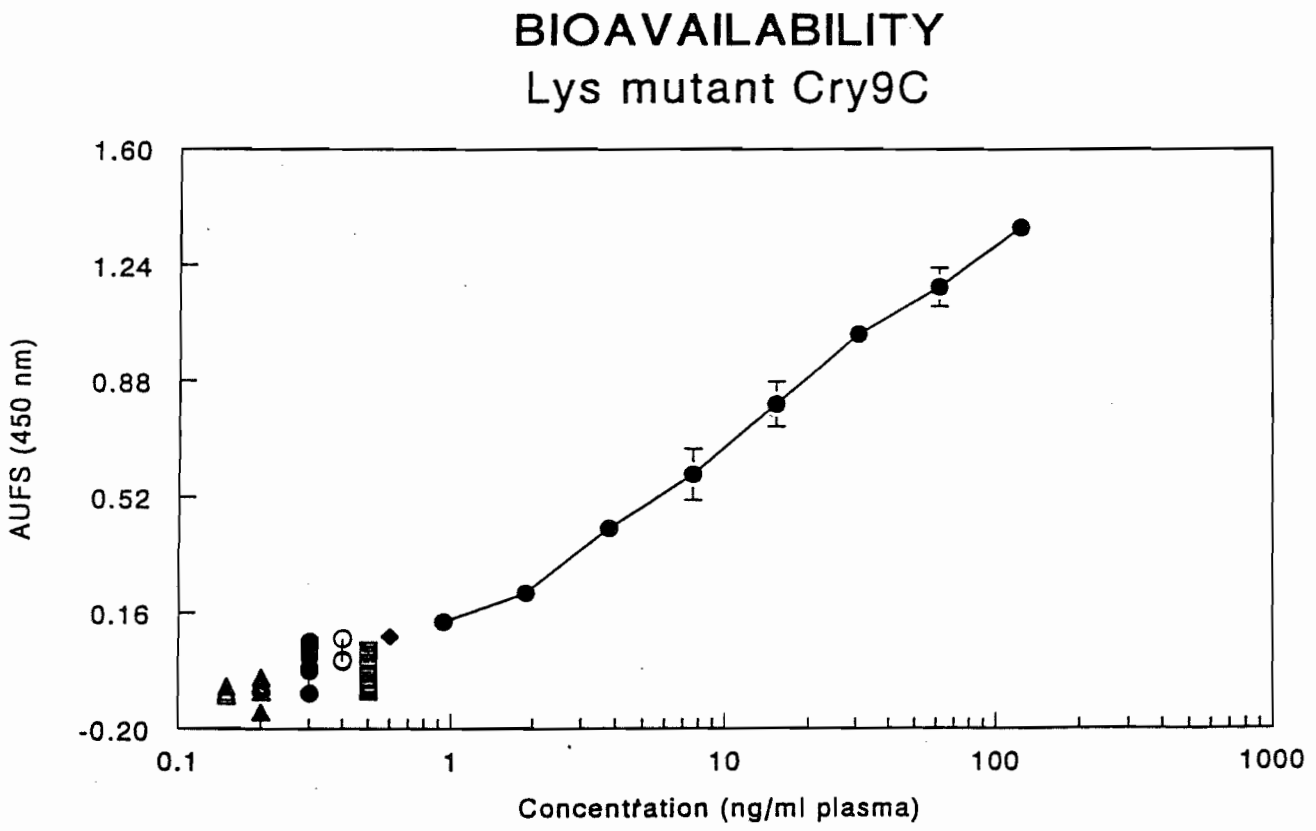


Figure 10.
Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein canulated rats (2.6 ± 0.1 mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 4 (i.e. the individual data points below the level of 1.0 ng/ml of plasma). Key: DS-ELISA standard calibration curve of Lys mutant Cry9C (i.e. black circles); rat no. 521 (open triangles); rat no. 522 (gray squares); rat no. 523 (gray circles); rat no. 524 (gray triangles); rat no. 525 (gray diamonds) and rat no. 528 (open circles). Detection limit: 0.05 AUFS or 0.8 ng/ml of plasma.

FIGURE 11

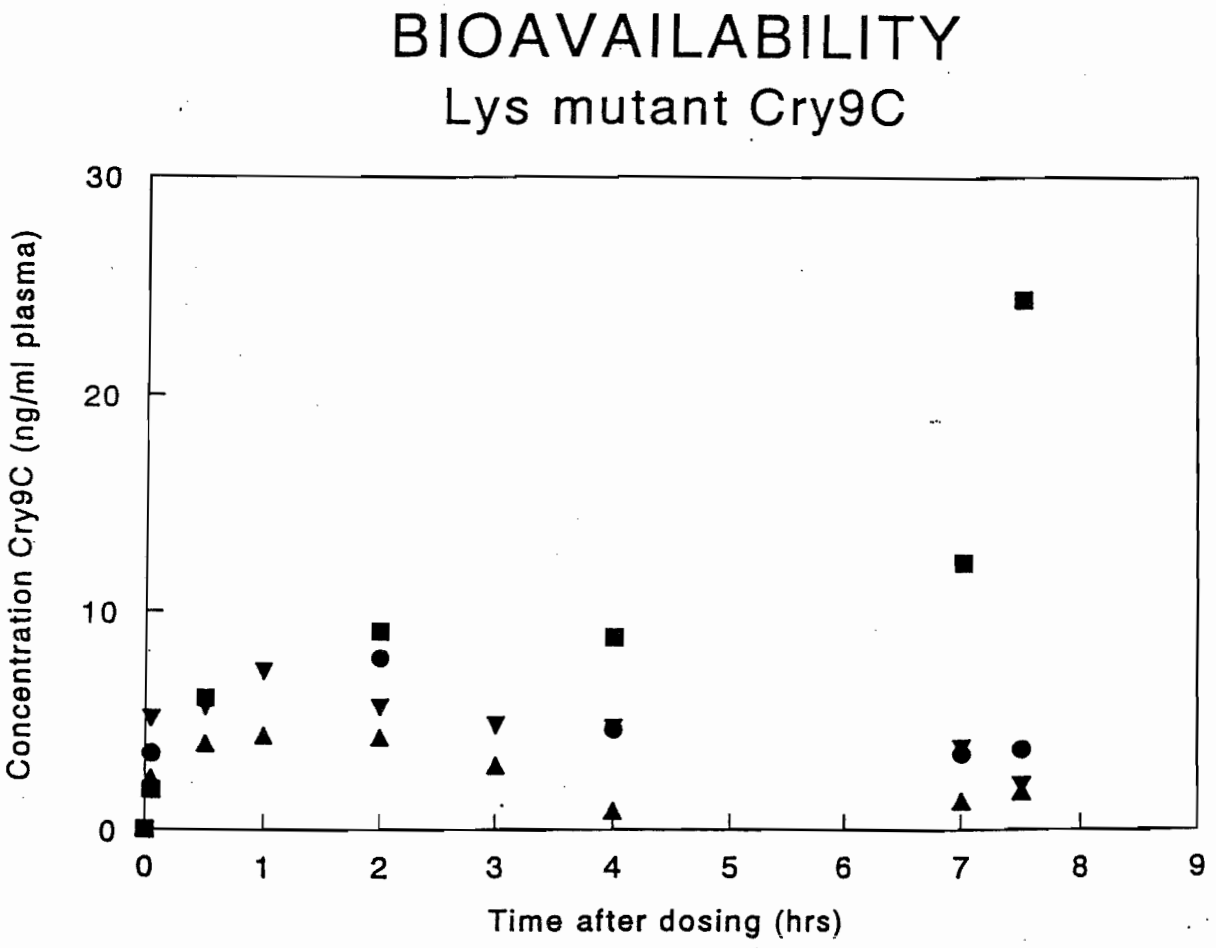


Figure 11.
Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein canulated rats (41.9 ± 1.8 mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 5. Key: rat no. 569 (closed circles); rat no. 573 (closed triangles); rat no. 575 (closed inverted triangles) and rat no. 576 (closed squares). Detection limit: 0.8 ng/ml of plasma.

057 24V

FIGURE 12

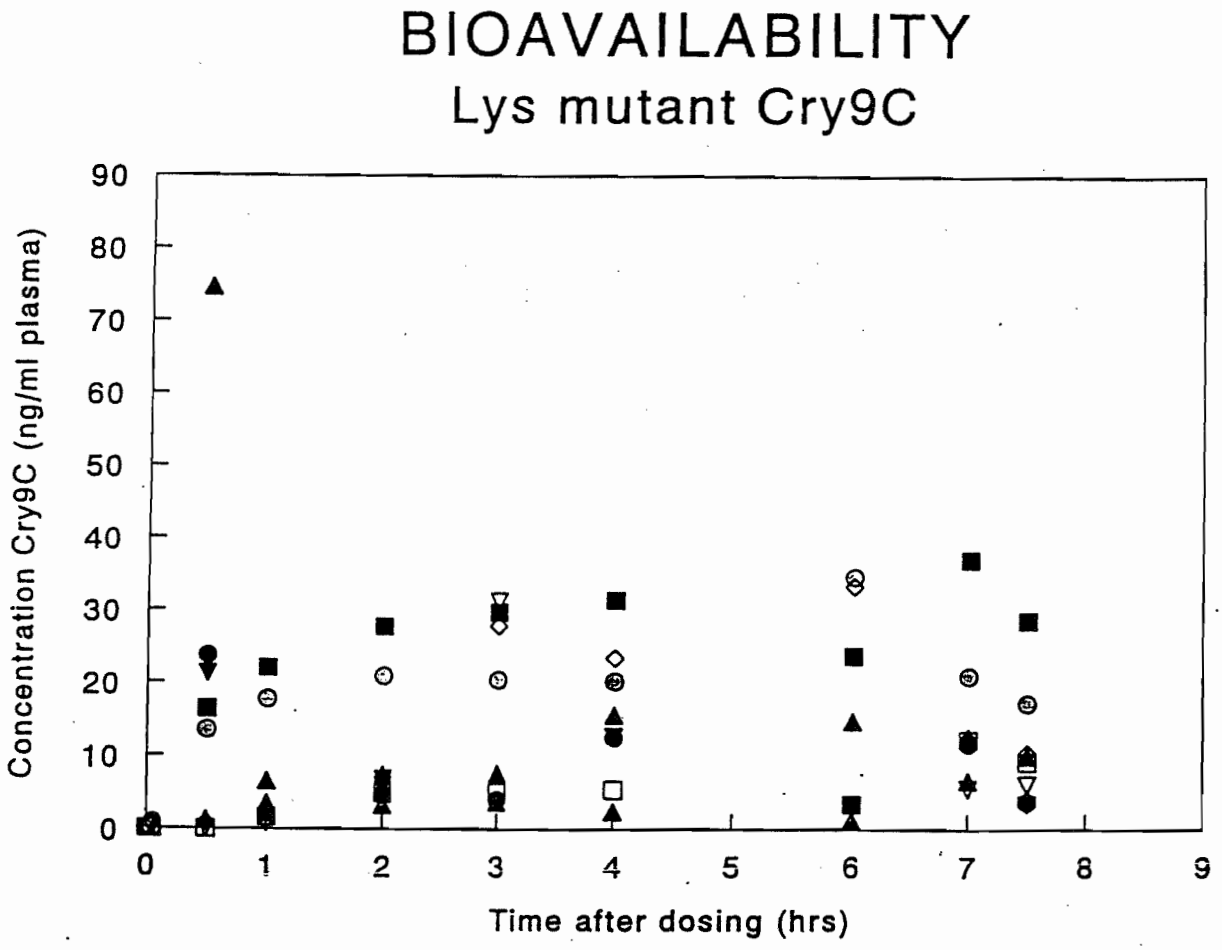


Figure 12.
Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein canulated rats (272 ± 27 mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 6. Key: rat no. 547 (closed circles); rat no. 548 (closed triangles); rat no. 549 (closed inverted triangles); rat no. 551 (closed squares); rat no. 556 (gray triangles); rat no. 558 (gray circles); rat no. 561 (open inverted triangles); rat no. 562 (open diamonds) and rat no. 550 (open squares). Detection limit: 0.8 ng/ml of plasma.

Figure 13.
Digestibility of the LYS mutant Cry9C protein upon G.I.-tract passage in rats as analyzed by western blotting (blot, gel no. 970716). Aliquots of gastric and luminal debris were collected at 8 hours after dosing and separated by SDS-PAGE and the molecular weight and stability of Cry9C was determined by immunoblot analysis. Rat nr. 550, 560, 561 and 551 samples taken from various gut compartments. Key: lanes s, Cry9C in stomic debris, lanes d, Cry9C in duodenal debris, lanes j, Cry9C in jejunal debris, lanes i, Cry9C in ileo debris, lanes c, Cry9C in caecal debris and lanes co; Cry9C in colonic/faecal debris. Lane Cry9C (stock solution, 4.4 ug/lane). The experimental conditions have been described in Annex 1 - Table 6.

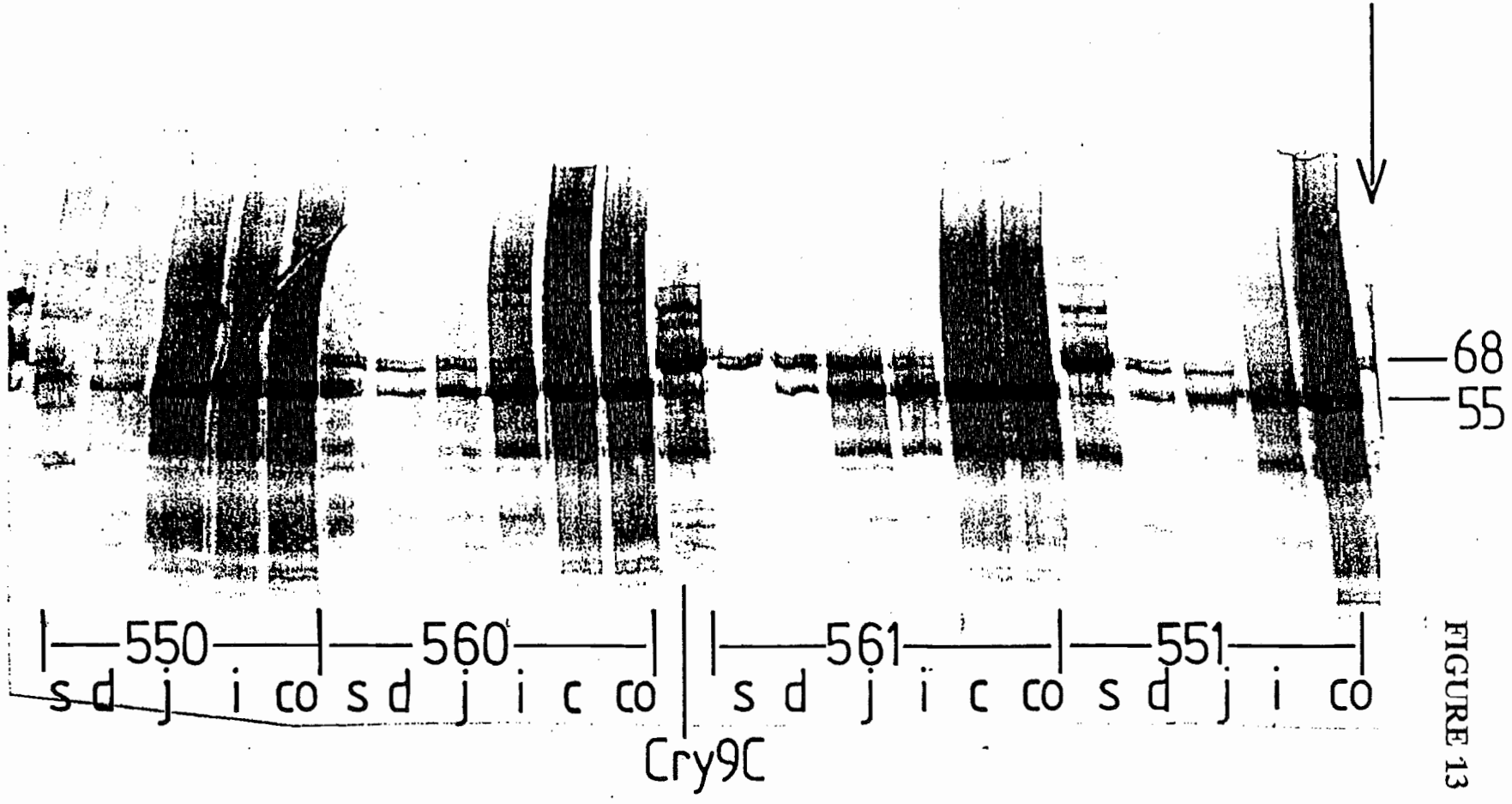


FIGURE 13

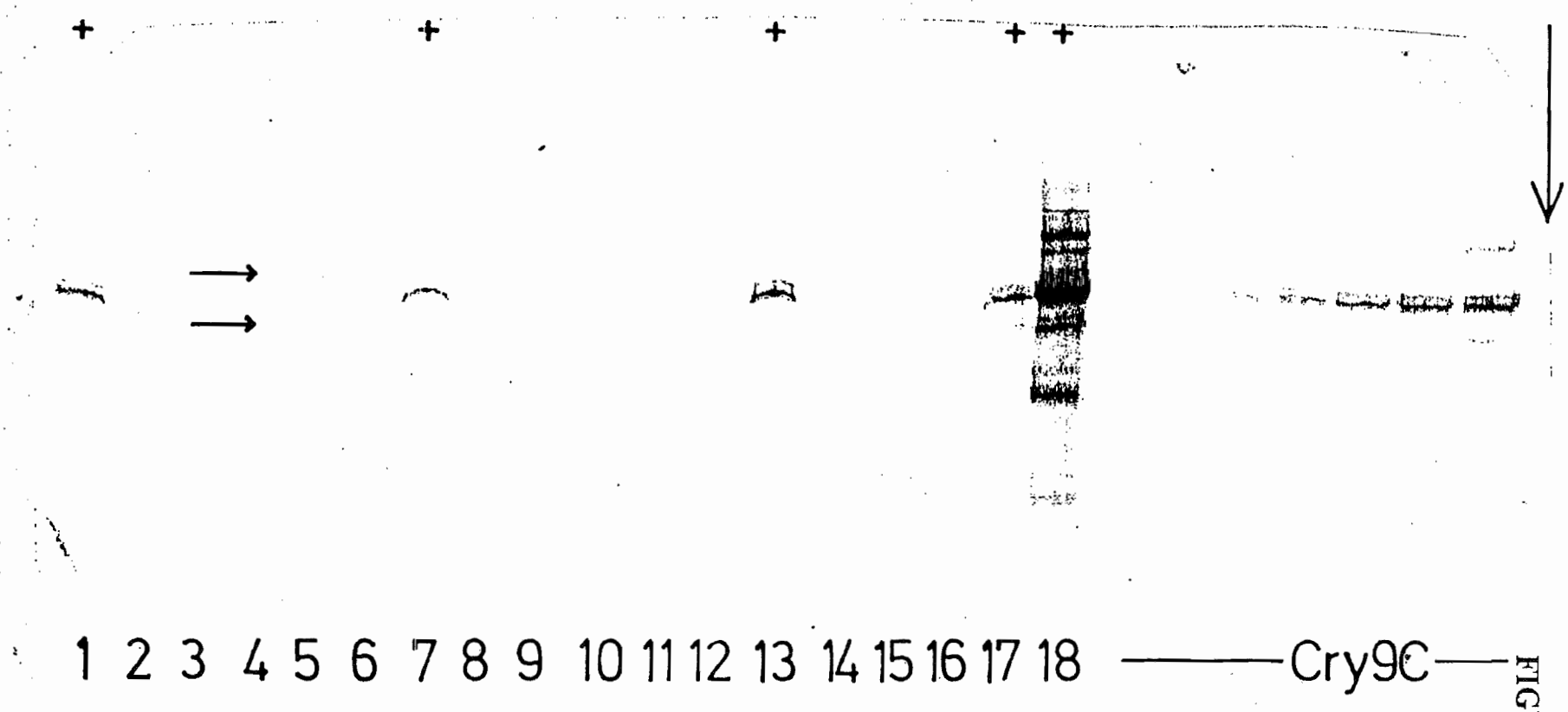


FIGURE 14

Figure 14.
Western blot analysis of the LYS mutant Cry9C protein immunoreactivity detected by DS-ELISA in the blood of rats (blot, gel no. 970820A). Aliquots of plasma (about 6 µl/lane) collected at various time points after dosing were separated by SDS-PAGE and the molecular weight of the Cry9C-like ELISA immunoreactivities determined by immunoblot analysis. Samples: lane 1 and 13, Cry9C (1.8 µg/lane), lane 7, Cry9C (0.9 µg/lane), lane 17, Cry9C (0.4 µg/lane) and lane 18, Cry9C (12.0 µg/lane); lane 2-4, plasma of rat 551 taken at 0 (lane 2), 7 (lane 3) and 7.5 (lane 4) hrs after dosing; lane 5-6, plasma of rat 548 taken at 0 (lane 6) and 0.5 (lane 5) hrs after dosing; lane 8-10, plasma of rat 567 taken at 0 (lane 10), 2(lane 9) and 7.5 (lane 8) hrs after dosing; lane 11-12, plasma of rat 558 taken at 0 (lane 12) and 6 (lane 11) hrs after dosing; lane 14-16, plasma of rat 562 taken at 0 (lane 16), 3 (lane 15) and 6 (lane 14) hrs after dosing. The experimental conditions have been described in Annex 1 - Table 6.

FIGURE 15

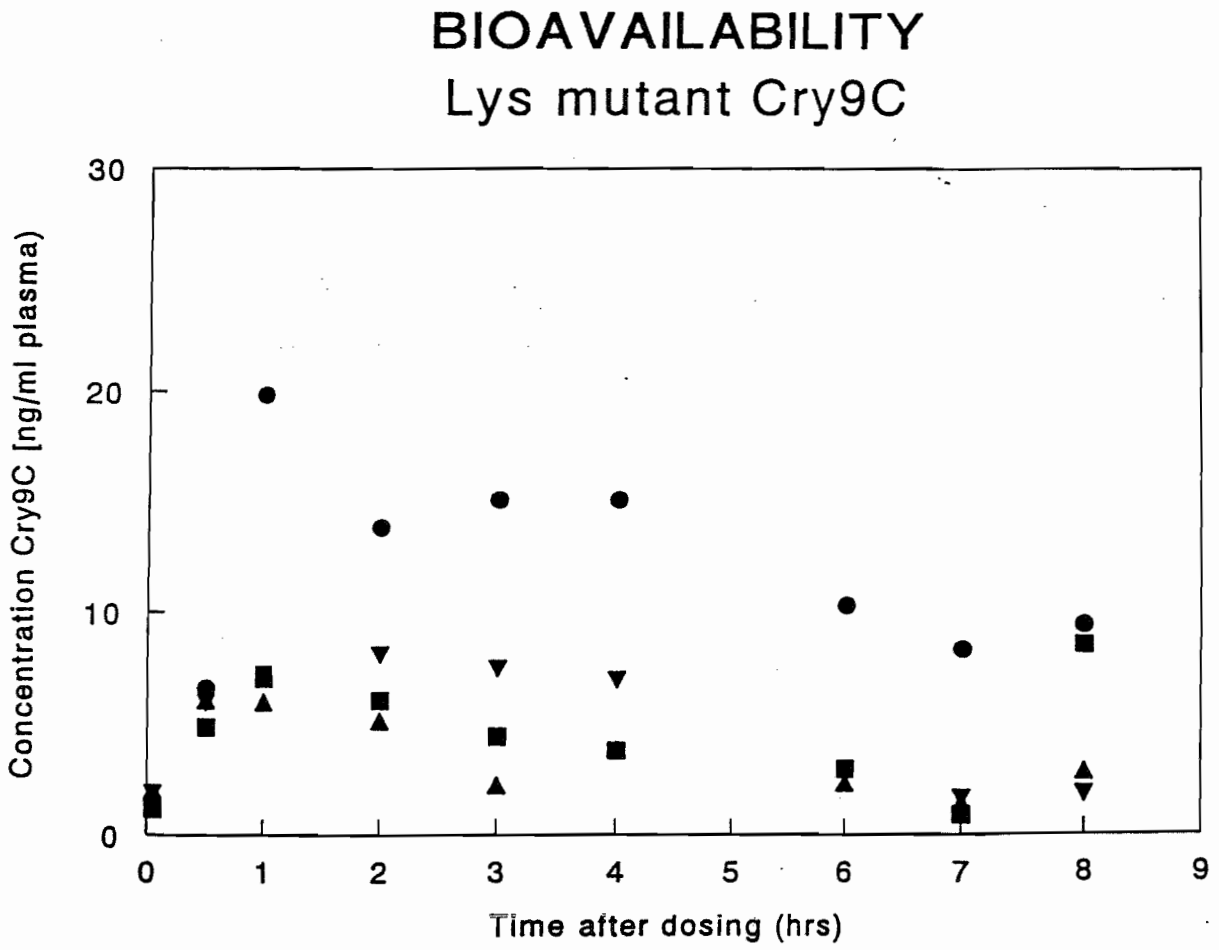


Figure 15.
Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein canulated rats (298 ± 6 mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 7. Key: rat no. 565 (closed circles); rat no. 564 (closed triangles); rat no. 574 (closed inverted triangles) and rat no. 568 (closed squares). Detection limit: 0.8 ng/ml of plasma.

FIGURE 16

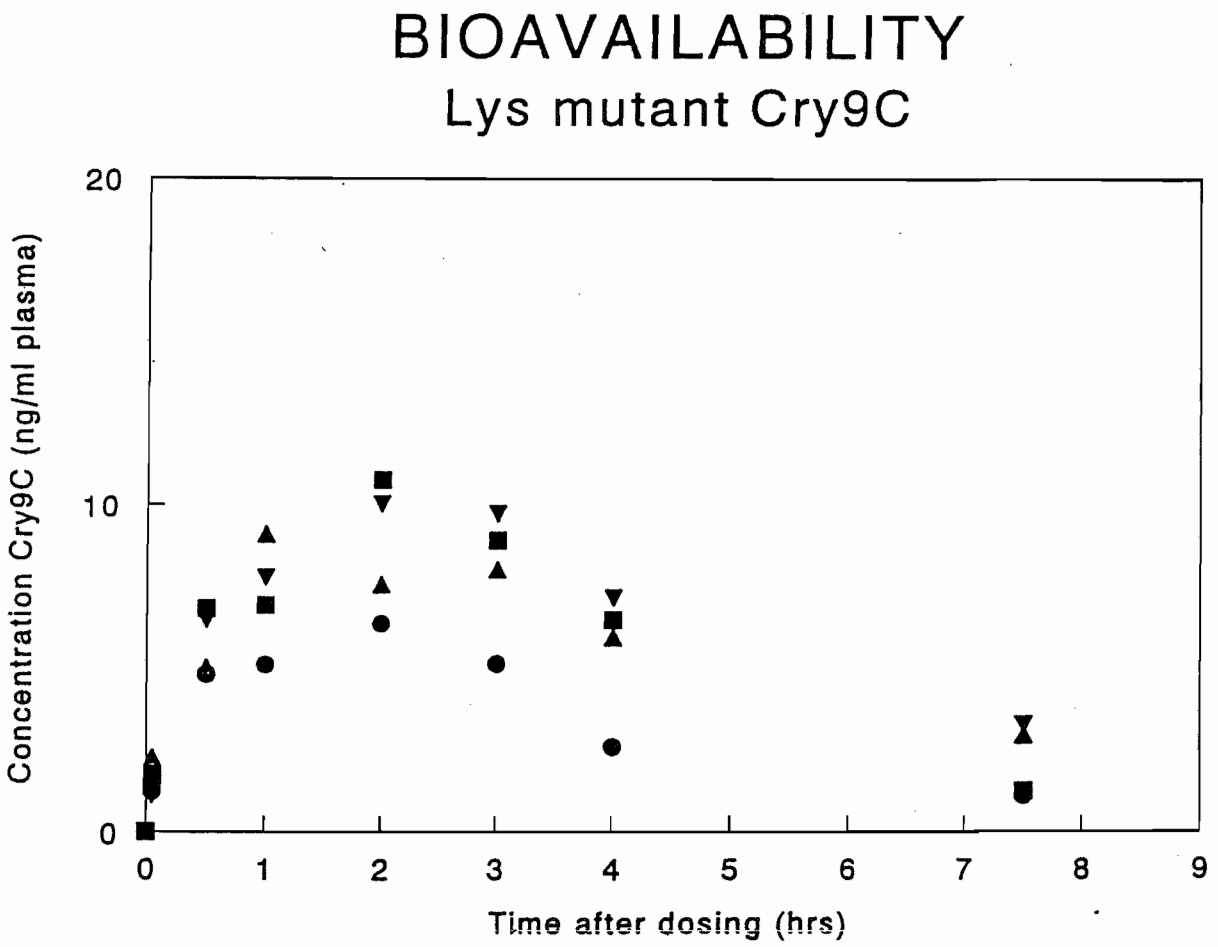


Figure 16.
Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein canulated rats (198.4 ± 2.9 mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 8. Key: rat no. 534 (closed circles); rat no. 532 (closed triangles); rat no. 531 (closed inverted triangles) and rat no. 530 (closed squares). Detection limit: 0.8 ng/ml of plasma.

n62 24 Aa

FIGURE 17

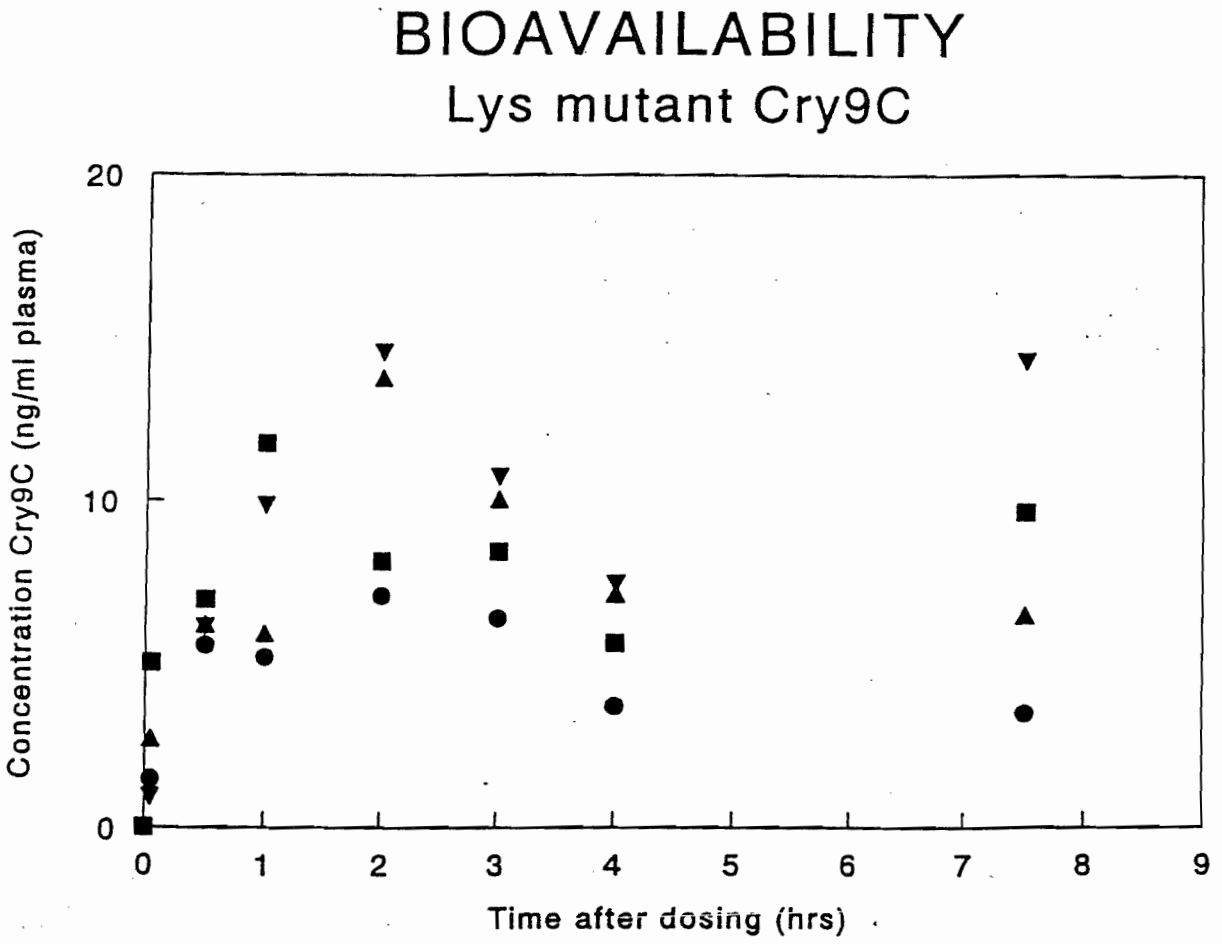


Figure 17.
Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein canulated rats (234.7 ± 8.1 mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 9. Key: rat no. 563 (closed circles); rat no. 577 (closed triangles); rat no. 567 (closed inverted triangles) and rat no. 570 (closed squares). Detection limit: 0.8 ng/ml of plasma.

563 24 Bb

FIGURE 18

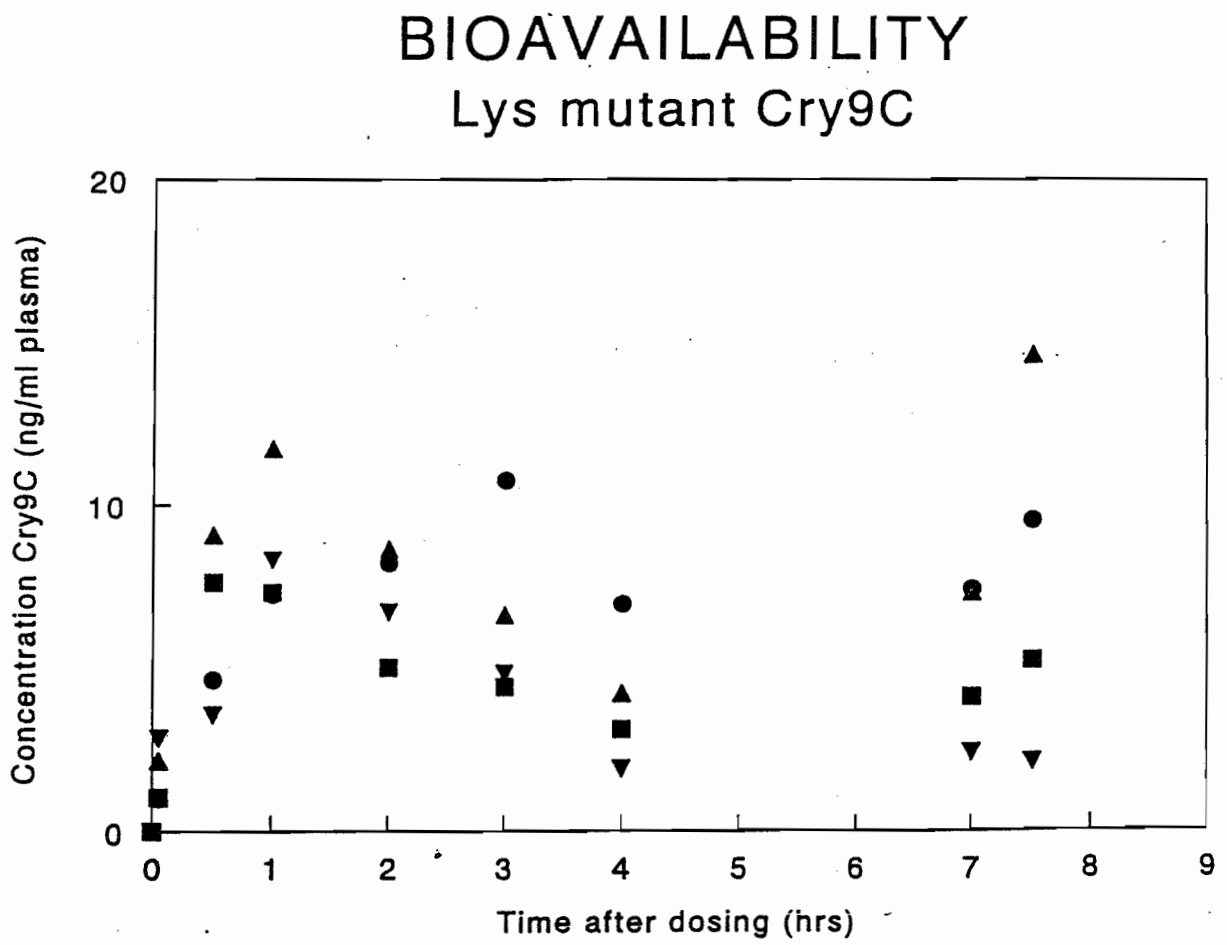


Figure 18.
Uptake of Lys mutant Cry9C protein versus time profiles after oral administration to portal vein canulated rats (229.0 ± 11.6 mg/kg body wt). The data represent values from the experiment as described in Annex 1 - Table 10. Key: rat no. 566 (closed circles); rat no. 571 (closed triangles); rat no. 572 (closed inverted triangles) and rat no. 578 (closed squares). Detection limit: 0.8 ng/ml of plasma.

n64 24cc

FIGURE 19

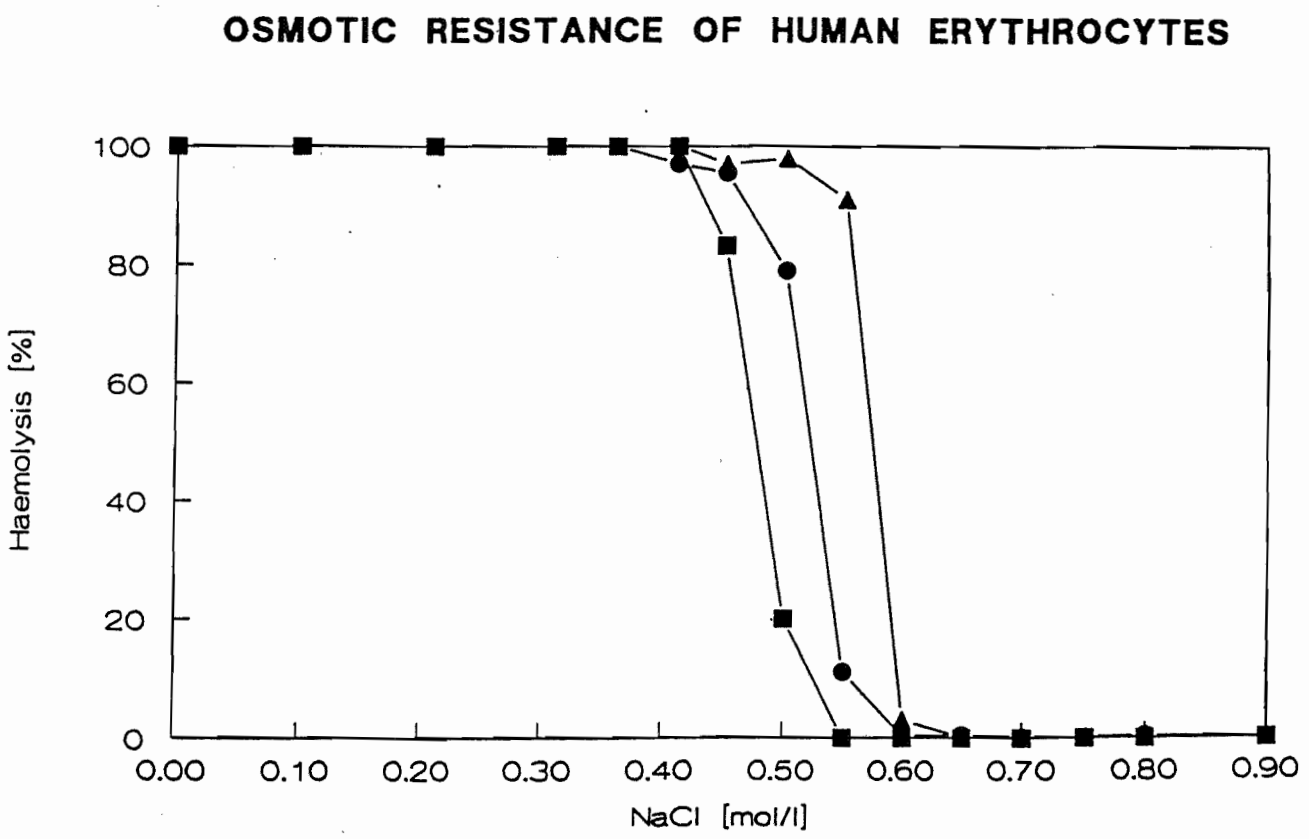


Figure 19.
Comparison of haemolysis in different NaCl solutions between human red blood cells in the presence of: (closed triangles) 0.5 mg/ml of Lys mutant Cry9C protein, (closed squares) 0.5 mg/ml of BSA and (closed circles) absence of proteins according to the method of Helleman et al. (1975).

065 24Dd

DATA EVALUATION REPORT

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

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

STUDY TYPE:	Protein Binding
MRID NO:	447343-01
TEST MATERIAL:	CRY9C Protein
PROJECT NO:	TOX 98012
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	AgrEvo UK Limited, Saffron Walden Essex, England & Plant Genetic Systems N.V., Gent, Belgium
TITLE OF REPORT:	BT CRY9C Protein: Investigative Study of the Potential for Binding to Mouse Intestinal Brush Membrane Vesicles
AUTHOR(S):	Konrad Purdy
STUDY COMPLETED:	November 5, 1998
CONCLUSION:	This study indicates that AgrEvo believes that the addition of unlabeled 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. Such a result would indicate that Cry9C binds specifically and saturably to *Ostrinia nubilalus* BBMVs and not to mouse intestinal BBMVs. However, the figures of the chemiluminescence results contained in the submission are of poor quality. Therefore, it is not possible to make an accurate assessment of the results without submission of the original, or much higher quality reproductions of the x-ray films. In addition, further discussion should be provided to support the conclusion drawn by AgrEvo regarding the displacement of labeled Cry9C proteins by unlabeled proteins. It is unclear from the data and discussion provided how such a conclusion has been reached.
CLASSIFICATION:	SUPPLEMENTAL. Can be upgraded to ACCEPTABLE with submission of adequate figures and supporting justification which correlate with the above-mentioned interpretation.
GOOD LABORATORY PRACTICE:	This study was performed in accordance with Good Laboratory Practice guidelines

195

I. STUDY DESIGN

Test Material: CRY9C protein derived from *Bacillus thuringiensis* subsp. *tolworthi* (batch # Cry9Ca-351-0198) and Cry1A(b) protein derived from *B. thuringiensis* subsp. *kurstaki*. Both proteins were produced in genetically altered *E. coli*.

Test Animals: Stock CD-1 mice were obtained from Charles River UK Ltd., Margate, Kent, UK. The animals were placed into cages in groups of five, after examination for any signs of ill health.

Methods:

Preparation of Biotinylated Cry9C:

Cry9C was incubated with Biotinyl-N-hydroxysuccinamide ester (BNHS). Following incubation, the biotinylated protein was separated from free BNHS using a Sephadex G-25 column.

Preparation of Insect Mid-Gut BBMVs:

Brush Border Membrane Vesicles (BBMVs) were prepared from fifth instar *Ostrinia nubilalus* larvae midguts by homogenization and ultracentrifugation for isolation of the vesicle fraction.

Preparation of Mouse Intestinal BBMVs:

Mouse intestinal BBMV's were prepared by eversion of intestines from CD-1 mice after sacrifice. The interior surface of the intestines was washed and scraped using a glass microscope slide. The scraped preparation was suspended in buffer (2.4 mM Tris, pH 7.1) containing Mannitol (60 mM) and EGTA (1 mM), homogenized and the vesicle fraction was isolated by ultracentrifugation. Chemical pathology assays were performed on the initial homogenate and the BBMV preparation to confirm isolation of the appropriate vesicle fraction. The chemical assays included:

- Total Protein
- Alkaline Phosphatase
- Gamma Glutamyl Transferase
- Leucine Aminopeptidase

Assessment of Binding of Cry9C to BBMVs:

BBMVs from insect mid-guts and mouse intestines were incubated for one hour at room temperature in each of the following mixtures:

Mixture A: 10 μg BBMV + 10 ng Biotinylated Cry9C

Mixture B: 10 μg BBMV + 10 ng Biotinylated Cry9C + 2 μg unlabeled Cry9C

The proteins were then separated using SDS-PAGE electrophoresis and electroblotted onto nitro-cellulose membranes. The membranes were incubated with streptavidin-horseradish peroxidase, followed enhanced chemiluminescence western blotting reagents (Amersham). The resulting chemiluminescence was detected using photographic emulsion.

II. RESULTS

Chemical Pathology - Assay results to confirm the correct isolation of the mouse intestinal BBMV fraction:

Fraction	Original Homogenate	BBMV Preparation
Total Protein (mg/ml)	1.76	0.47
Leucine Aminopeptidase (U/mg protein)	90	479
Alkaline Phosphatase (U/mg protein)	998	6464
γ-Glutamyl Transferase (U/mg protein)	44	232

The results indicate that the enrichment of the BBMV fraction is approximately 5-fold compared to the original homogenate.

Binding of Cry9C to BBMVs:

Insect BBMVs - A distinct band of the expected molecular weight (70 kDa) was detected from Mixture A and an extremely faint band was detected from Mixture B showing, according to AgrEvo, that the addition of the unlabeled Cry9C into the incubation solution resulted in displacement of biotinylated Cry9C.

Mouse BBMVs - A band of the expected molecular (70 kDa), and an additional band of lower molecular weight were detected from Mixture A. A band of the expected molecular weight was detected from Mixture B, showing that the addition of unlabeled Cry9C into the incubation solution, did not result in the displacement of the biotinylated Cry9C, again according to AgrEvo.

III. DISCUSSION

The study author indicates that the addition of unlabeled Cry9C in the displacement of the labeled (biotinylated) Cry9C, demonstrating that the Cry9C binds specifically and saturably to *Ostrinia nubilalus* BBMVs. In addition, the addition of unlabeled Cry9C did not result in the displacement of the labeled Cry9C, demonstrating that Cry9C shows only a small amount of non-specific binding, and does not bind specifically and saturably to mouse intestinal BBMVs.

However, the copy of the electrophoretogram provided is not of very good quality, and therefore, it is impossible for BPPD to reach these same conclusions. The picture of the insect BBMVs has a very dark background, and it is not possible to determine if more than one band is present, specifically the "faint" band that would represent Mixture B. Also, the picture of the mouse BBMVs has virtually no background, and therefore it is also not possible to determine if additional "faint" bands might be present. Neither of the figures have molecular weight markers to

verify the 70 kDa weight of the proteins or control samples for comparison. The original, or a much higher quality copy of these films, should be provided in order for an accurate assessment to be made regarding these conclusions. In addition, there is no explanation provided, nor a molecular weight for the additional band present in the mouse samples. This information should also be provided.

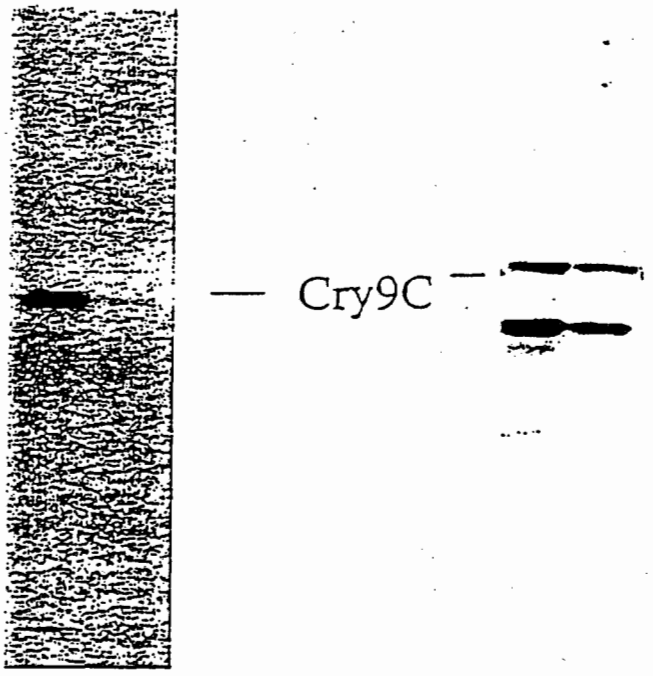
Assuming that clear figures are available, it is also unclear to BPPD what AgrEvo means by "displacement" by the unlabeled Cry9C in the insect BBMV samples. The term "displacement" indicates an active process where there is a mechanism responsible for dislodging labeled proteins and allowing these displaced proteins to be replaced by the unlabeled Cry9C proteins, or some similar process. There is no discussion in this submission which addresses the displacement issue. Further explanation/discussion should be provided.

CLASSIFICATION: SUPPLEMENTAL. This submission can be upgraded to ACCEPTABLE with submission of adequate figures and additional information to support the conclusion reached regarding the binding potential of Cry9C to the tissues tested. Supplemental data/information should include:

1. Adequate figures (originals or high quality reproductions) of the chemiluminescent results that will allow for clear differentiation between protein signals and background "noise".
2. Molecular weight markers to clearly identify the molecular weights of the identified protein bands. Also, identification of any "control" protein samples used for comparison to test samples.
3. Explanation/identification of the additional bands present in the Mouse BBMV samples.
4. Further discussion and justification regarding the "displacement" of the labeled Cry9C proteins by the unlabeled proteins.

APPENDIX

ELECTROPHORETOGRAMS SHOWING LABELLED CRY9C FOLLOWING INCUBATION WITH INSECT AND MOUSE BBMVs



Labelled Cry9C	:	+	-	+	+
200 fold unlabelled Cry9C	:	-	-	-	+

Key:

ECB = Insect BBMVs	MOUSE = Mouse BBMVs
1. = Mixture A	3. = Mixture A
2. = Mixture B	4. = Mixture B

DATA EVALUATION REPORT

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

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

STUDY TYPE:	Acute Intravenous Toxicity (152A-13)
MRID NO:	447343-02
TEST MATERIAL:	*Bt* Cry9C and *Bt* Cry1A(b) Proteins
PROJECT NO:	TOX 98009
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	AgrEvo UK Limited, Saffron Walden Essex, England
TITLE OF REPORT:	Bt Cry9C Protein Mouse Acute Intravenous Toxicity Study
AUTHOR(S):	N. Buss, M. McFarlane
STUDY COMPLETED:	October 7, 1998
CONCLUSION:	Male CD-1 mice were dosed with saline solutions containing CRY9C, CRY1A(b) and BSA proteins. The animals were observed for clinical effects over a 14 day period, and were examined by gross necropsy at the end of the observation period. A dose of 0.3 mg/kg body weight did not produce any apparent adverse effects in the mice tested.
CLASSIFICATION:	ACCEPTABLE
GOOD LABORATORY PRACTICE:	This Study Was Performed in Accordance with Good Laboratory Standards guidelines.

I. STUDY DESIGN

Test Material: *Bt* Cry9C protein derived from *Bacillus thuringiensis* subsp. *tolworthi* and *Bt* Cry1A(b) protein derived from *B. thuringiensis* subsp. *kurstaki*, obtained by expression in *E. coli*. Control test material was bovine serum albumin (BSA), obtained from Sigma Aldrich Fluka.

Test Animals: Forty-five male CD-1 mice were received from Charles River UK Ltd., with body weights in the range of 16.5 g to 21.2 g. The animals were allowed one day of acclimatization, examined, and separated into four groups of five mice on the basis of body weights. The groups of mice had similar initial mean body weights and weight distributions, with a range between 21.3 and 22.8g.

Methods: Body Weights: Each animal was weighed upon receipt, at separation into groups, prior to the start of treatment, on Days 1 & 8, and at termination.

Dosing: The theoretical maximum daily intake (TMDI) was calculated based upon the assumption that the only crops containing Cry9C protein are maize, cotton, and rape and that the maximum levels present are estimated to be c. 18 ppm (18 mg/kg/day). Based upon this, the global TMDI was calculated to be 0.0247 mg/kg/day and based upon the European diet, the TMDI would be 0.0169 mg/kg/day.

AgrEvo has determined the possible human systemic exposure resulting from an oral exposure of an estimated TMDI (0.03 mg/kg/day) to be 0.2 ng/kg/day based on information from a bioavailability study in rats. From this calculation, the intravenous dose level chosen was 0.3 mg/kg, which provided a 150,000 fold margin of safety over the estimated systemic human level. Both BSA and CRY 1A(b) proteins were administered at the same dose level to allow for direct comparison.

Table 1. Treatment Groups

Group Number	Test Material	Dose Level (mg/kg)	Cage Number	Animal Number
1	0.9% Sodium Chloride (vehicle)	0	1	7631-7635
2	Bt Cry 9C	0.3	2	7636-7640
3	Bt Cry 1 A(b)	0.3	3	7641-7645
4	Bovine Serum Albumin	0.3	4	7646-7650

The test material was administered intravenously (in sterile 0.9% sodium chloride) through the tail vein at a constant volume of 10 ml/kg based upon body weights using in-line 0.2μm syringe filters to ensure sterility.

All surviving animals were euthanized on Day 14.

Clinical Signs: Observations were made frequently on the day of treatment, and at least once each morning thereafter. The animals were also observed on Monday and Friday afternoons.

Chemical Pathology: Blood samples for analysis of Cry9C and Cry 1A(b) proteins were collected on the day of necropsy (Day 14). All samples were obtained from the retro-orbital sinus under isoflurane anaesthesia. The blood samples were then snap frozen in liquid nitrogen at -70° C and sent for storage at Plant Genetic Systems.

28E

Necropsy: Surviving animals were euthanized by carbon dioxide asphyxiation on Day 14 and subjected to gross *post-mortem* examination.

II. RESULTS

Mortality: No animals died during the 14 day observation period.

Clinical Signs: No treatment-related clinical signs were observed through the 14 day observation period.

Body Weight: None of the animals lost weight over the 14 day observation period. One animal in the BSA test group did not gain as much weight as the other animals in this, and other groups, and therefore the mean for this group was significantly different from the control. However, this result did not affect the outcome of the testing.

Gross Necropsy: There were no abnormal findings in any of the animals examined by gross necropsy at the end of the 14 day observation period.

Chemical Pathology: No analysis of these samples was reported.

III. DISCUSSION

The test substances used in these tests were proteins derived from two *B. thuringiensis* species and BSA. In the toxicity testing included in this submission, there does not appear to be toxic effects associated with any of the three proteins. A 14 day observation period after dosing did not reveal any abnormalities associated with injection of any of the proteins.
CLASSIFICATION: ACCEPTABLE

DATA EVALUATION REPORT

Reviewed by: Michael T. Watson, Ph.D., Plant Pathologist MTW
Secondary Reviewer: John L. Kough, Ph.D., Biologist JLK

STUDY TYPE:	Protein Stability
MRID NO:	447343-04
TEST MATERIAL:	Cry9C Protein
PROJECT NO:	CM-97B-01
SPONSOR:	AgrEvo USA Company, Wilmington, DE
TESTING FACILITY:	AgrEvo USA Company, Pikeville, NC
TITLE OF REPORT:	Determination of the Stability of PAT and Cry9C Protein in Processed Grain of Transgenic Field Corn in Fractionated Agricultural Commodities
AUTHOR(S):	Raymond D. Shillito
STUDY COMPLETED:	January 13, 1999
CONCLUSION:	Results of ELISA tests, using antibodies specific for PAT and CRY9C proteins suggest that these proteins are present in transgenic corn plants, in relatively small amounts as a percentage of total protein. However, these results are somewhat questionable due to the results obtained from control corn grown in Illinois. The control corn, grown in a location adjacent to the transgenic corn, also showed positive results for both the PAT and Cry9C proteins. Although the amount of protein in the controls is small, compared to the transgenic lines, this result is surprising. From the data provided, it not possible to determine why the controls grown in Illinois provided these results. It appears that there was possibly contamination during the processing of the corn samples. There was no Cry9C protein detected in the control whole corn samples, yet the protein was detected in several of the processed samples from this same corn. Therefore, the results provided for both the control and test samples become somewhat questionable. Although it is likely that the Cry9C and PAT proteins are present in relatively small amounts in the transgenic plant line, further justification/explanation should be provided to address the issue of positive reactions in the control samples, and what impact this has upon the data for the transgenic corn fractions.
CLASSIFICATION:	SUPPLEMENTARY. This submission can be upgraded to ACCEPTABLE with submission of an adequate justification or supplemental data for the results in the control plants.
GOOD LABORATORY PRACTICE:	The field study portion of this study was not performed in accordance with Good Laboratory Standards guidelines.

I. STUDY DESIGN

Test Material: Protein Reference Samples -

- PAT Protein (Phosphinothricin-N-Acetyltransferase)
- Cry9C Protein (Insecticidal Crystal Protein 9C)

Both proteins, and the antibodies specific for each protein were supplied by Plant Genetic Systems.

Plant Samples -

Illinois Trial - Transgenic glufosinate resistant and corn-borer resistant *Bt* field corn containing the *bar* and *cry9C genes* (CHB351), and near isogenic non-transgenic, non-resistant corn. The trial was harvested at maturity on 10/31/97.

North Carolina Trial - Non-transgenic, non-resistant corn plants (Pioneer Hybrid 3394). The trial was harvested at maturity on 10/7/97.

Methods:

A. Processing - Processing was carried out under GLP at the Food Protein Research and Development Center, (Texas A & M). SOP numbers 8.6 (revision 08) and 8.5 (revision 09) were followed (attached). Samples of the whole corn were removed and frozen for analysis before processing. Wet milled commodities produced were: hulls (bran), steepwater concentrate, gluten, starch, crude oil, refined oil, and solvent extracted germ (presscake). Dry milled commodities produced were: hulls (bran), grits, meal, flour, crude oil, refined oil, and solvent extracted germ.

Illinois Trial: Approximately 369 pounds of control corn and 42 pounds of transgenic corn were processed. Samples were frozen immediately after processing and sent frozen to AgrEvo laboratories for analysis.

North Carolina Trial: Approximately 580 pounds of control corn were processed. Samples were immediately frozen after processing and sent to AgrEvo labs for analysis.

2. PAT and Cry9C Protein Analysis -

The presence and amount of both the Cry9C and PAT proteins was determined by ELISA using PAT or Cry9C-specific antibodies in samples of grain and processed fractions from transgenic and non-transgenic corn plants. The ELISA test for each protein is capable of detecting both intact and degraded proteins. The test and validation samples were generated by studies CM97B01 and BK97B04, respectively.

Seed, grits, hulls, and solvent extracted germ were ground in the presence of dry ice before extraction for ELISA assay. Further processing was not necessary for meal, crude and refined oil and flour. Non-transgenic and transgenic samples were ground on different days. Standards and non-transformed samples fortified with pure Cry9C or PAT protein prior to extraction were included with each set of assays.

A representative sample (approximately 1 g) was mixed with the extraction buffer (10 ml) in a 50 ml centrifuge tube, shaken for 30 minutes (@ 4° C, 700 rpm) and

2914

then centrifuged at 4190 g for 10 minutes. The supernatant was transferred to a clean tube and the cycle of centrifugation and separation was repeated to produce a clear supernatant (duplicate extracts were prepared for each sample).

The total extractable protein (TEP) was determined for each sample extract. Duplicate 10 μl aliquots of the sample extract were placed onto microtiter plates, followed by addition of 200 μl of Bradford Reagent. The samples were incubated for 15-20 minutes on a shaker and the OD was measured (595 nm)

3. Limit of Detection -
A set of eight standards ranging from 0 to 30 ng/ml of PAT or Cry9C were included in duplicate on each respective ELISA plate. The limit of detection (LOD) for each matrix using the optical density (OD) of the control samples based on the 0.95 confidence level in one tail t-distribution:

$$OD_{LOD} = OD_{mean} + [(t_s \times SD) / (n-1)^{0.5}]$$

OD_{LOD} = optical density corresponding to the LOD
OD_{mean} = mean OD of the zero dose replicates
N = number zero dose replicates
T_s = t critical value for a one-sided test at p = 0.95 and df = n-1
p = probability or confidence level
df = degree of freedom

The ELISA reading above this limit of detection can be assumed to represent a 95% probability of being greater than zero dose reading.

4. Limit of Quantitation -
The LOQ (limit of quantitation) is given by the lowest concentration of the standard (0.47 ng/ml) or the LOD when this value is greater than the lowest concentration of standard (Table 1). Values below LOQ are reported as non-detectable (ND).

Table 1. Limits of Quantitation of PAT and Cry9C Proteins in Processed Commodities of Field Corn as Detected by ELISA

Process	Commodity	PAT ELISA LOQ (ng/ml)	Cry9C ELISA LOQ (ng/ml)
	Whole Corn	2.01	0.47
Dry Mill	Composite Grits	2.50	0.47
	Meal	0.47	0.47
	Flour	0.47	0.47
	Hull Material	0.47	0.47
	Solvent Extracted Germ	6.40	0.47
	Crude Oil	0.80	0.47
	Refined Oil	0.47	0.47
Wet Mill	Steepwater Concentrate	0.82	0.47
	Hull Material	0.47	0.47
	Gluten	0.47	0.47
	Starch	0.47	0.47
	Solvent Extracted Germ	0.47	0.47
	Crude Oil	0.47	0.47
	Refined Oil	0.47	0.47

E. Validation -

The PAT and Cry9C ELISA procedures were validated for whole corn and processed corn samples using the PAT and Cry9C standards. Due to what AgrEvo believes was apparent contamination of the control sample from this study (CM97B01 - grown in Champaign County, Illinois), determination of LOD and LOQ's and validation were carried out using the control samples from another study (BK97B04) which was conducted in Wayne County, North Carolina.

Non-transgenic control samples were separately fortified at 0.9 ng/ml and 30 ng/ml with either PAT ELISA in four replicates, or with Cry9C ELISA in six replicates. The fortified samples were processed in extraction buffer prior to the extractions. Each replicate was analyzed using duplicate wells.

4914

II. RESULTS

A. Protein Content:

Results of quantitation of Cry9C and PAT proteins from transformed field corn (CBH351) are shown in Table 2 below. Cry9C was found in all samples except for the crude and refined oil samples. The protein content ranged between 66.3 and 32573 ng/g. PAT protein was found in all samples except for crude oil, refined oil gluten, and starch fractions. The protein content ranged between 33 and 82184 ng/g.

Table 2. Quantities of PAT and Cry9C in Processed Commodities of Transformed Field Corn CBH 351 from the Illinois Trial as Detected by ELISA

Process	Commodity	PAT ELISA (ng/g sample)	Cry9C ELISA (ng/g sample)
	Whole Corn	14795 ± 897*	12287 ± 680
Dry Mill	Composite Grits	11326 ± 957	9506 ± 1229
	Meal	5317 ± 201	10720 ± 745
	Flour	2160 ± 167	8408 ± 735
	Hull Material	2363 ± 130	6426 ± 285
	Solvent Extracted Germ	82184 ± 13868	32573 ± 2828
	Crude Oil	ND	ND
	Refined Oil	ND	ND
Wet Mill	Steepwater Concentrate	ND	1802 ± 170
	Hull Material	33 ± 6014	3488 ± 443
	Gluten	ND	1155 ± 45
	Starch	ND	66.3 ± 5.1
	Solvent Extracted Germ	1901 ± 14.0	8800 ± 698
	Crude Oil	ND	ND
	Refined Oil	ND	ND

* ± Standard Deviation (each data point is the average of two assays each performed on two subsamples)

ND = Not Detectable (i.e. below the LOQ for the matrix)

Both Cry9C and PAT proteins were found in the control samples grown at the Illinois site. The levels of each of the proteins was less than 10% of that found in the transgenic corn samples, but not expected for a non-transformed control. AgrEvo indicates in their submission that examination of data for growth, shipping

5814

and processing of the samples did not reveal an explanation for the presence of these protein in the controls. Therefore, the validation assay was carried out using a control plot grown in North Carolina [BK97B04 - transgenic glufosinate resistant field corn containing the *pat* gene (T25) and near isogenic non-transgenic, non-resistant corn plants - Appendix].

Table 3. Quantities of PAT and Cry9C proteins in Processed Commodities of Control Material from the Illinois Trial as Detected by ELISA

Process	Commodity	PAT ELISA (ng/g sample)	Cry9C ELISA (ng/g sample)
	Whole Corn	ND	ND
Dry Mill	Composite Grits	188 ± 15.4	231 ± 60.5
	Meal	164 ± 27.6	297 ± 11.2
	Flour	62 ± 2.69	274 ± 29.8
	Hull Material	200 ± 8.18	296 ± 24.2
	Solvent Extracted Germ	1640 ± 137	1613 ± 100
	Crude Oil	ND	ND
	Refined Oil	ND	ND
Wet Mill	Steepwater Concentrate	ND	146 ± 9.58
	Hull Material	ND	102 ± 4.69
	Gluten	ND	5.03 ± 0.67
	Starch	ND	ND
	Solvent Extracted Germ	7 ± 1.96	5.25 ± 2.51
	Crude Oil	ND	ND
	Refined Oil	ND	ND

* ± Standard Deviation (each data point is the average of two assays each performed on two subsamples)

ND = Not Detectable (i.e. below the LOQ for the matrix)

6 of 14

The quantities of Cry9C and PAT proteins were expressed as a percentage of total crude protein found in the respective fractions (Table 4).

Table 4. Quantities of PAT and Cry9C Proteins in Processed Commodities of Transformed Field Corn CBH 351 Expressed as Percent of Crude Protein

Process	Commodity	Crude Protein in Matrix (%)*	PAT Protein as % Crude Protein	Cry9C Protein as % Crude Protein
	Whole Corn	8.910	0.0166	0.0138
Dry Mill	Composite Grits	7-10.3	0.0162	0.0136
	Meal	7.5-9.0	0.0071	0.0143
	Flour	5.2-7.8	0.0042	0.0161
	Hull Material	8	0.0030	0.0080
	Solvent Extracted Germ	12-25	0.0685	0.0271
	Crude Oil	0	-	-
	Refined Oil	0	-	-
Wet Mill	Steepwater Concentrate	41-62	-	-
	Hull Material	8	0.00004	0.0043
	Gluten	41-60	-	-
	Starch	0.6	-	-
	Solvent Extracted Germ	22.6	0.000084	0.0039
	Crude Oil	0	-	-
	Refined Oil	0	-	-

* Range of data from CRC, Vol II, 1982; Ensminger et al. 1990; McGregor 1994.

B. Validation Study:

When fortified at 0.9 ng/mg, PAT protein was not quantifiable in whole corn, composite grits, flour, solvent extracted germ, crude oil, refined oil, steepwater concentrate, hull material (wet mill) and starch. The mean recoveries for other matrices ranged between 75.3% to 99.3% depending on the matrix (Table 4 - Appendix). When fortified at 30 ng/ml, the mean recoveries of PAT protein were between 27 and 166% among different matrices with the exception of refined oil (wet mill).

When fortified at 0.9 ng/ml Cry9C protein was not quantifiable in composite meal, flour, hull material, steepwater concentrate, gluten, starch, solvent extracted germ press cake, crude oil, and refined oil. The mean recoveries for other matrices range from 58.2 to 130% (Table 5. - Appendix) depending on the matrix. When fortified at 30 ng/ml, the mean recoveries of PAT protein were between 20.4 and 89.8% among the different matrices with the exception of refined oil precessed by we mill. The low recovery of PAT protein from refined oil can be attributed to the denaturation of protein caused by oil-aqueous phase mixing.

C. Total Extractable Protein:

The protein contents in non-transgenic corn grains and processed fraction are shown in Table 6 (Appendix). The total extractable protein (TEP) varied from 0 to 30.3 mg/g. PAT protein was found in composite grits, composite meal, flour, hull material (dry mill) solvent extracted germ, steepwater concentrate, hull material (wet mill), gluten and solvent extracted germ press cake .

The protein contents in line the transgenic line (CBH351) fractions are shown in Table 7 (Appendix). The total extractable protein varied from 0 to 21.5 mg/g. PAT protein was found in whole corn, composite grits, composite meal, flour, hull material (dry mill), solvent extracted germ, hull material (wet mill), and solvent extracted germ press cake. Cry9C protein was found in whole corn, composite grits, composite meal, flour, hull material (dry mill), gluten, starch and solvent extracted germ press cake.

III. DISCUSSION

The data presented in Tables 2, 3 & 4 indicate the amount of the PAT and Cry9C proteins present in the respective parts of the corn and corn products. Table 4 provides relative information regarding the amounts of each of these proteins, and their amounts as a percentage of total proteins in the representative materials. Overall, based upon the data provided, these proteins are present at a maximum percentage of 0.0685% (dry mill - solvent extract germ), representing a relative small amount of total protein.

However, these data are somewhat questionable due to the levels of proteins found in the control samples grown in Illinois. It is certainly odd that both proteins are found in many of the control samples. It is possible that these results are simply the result of contamination of the control corn samples either in the field, or during the processing phase of the study. However, based upon the data provided, it is not possible to rule out the possibility that there was expression of the Cry9C and PAT proteins in the control corn. This scenario is not likely because the proteins were not detected in the control whole corn samples. But, because of positive signals in

8.9.14

the control samples, there is no definitive means to conclude that the results of the transgenic fractions are not flawed. The validation assay was carried out using samples from a control plot of Glufosinate resistant corn grown in North Carolina (1997). The data and analysis from this study appear to be adequate, however, because the control was a different line of corn, grown in a different state under different (unidentified) growth conditions, these data can also be considered questionable in their relevance to this study. In addition, because they do not address the issue of why the control samples gave positive results for the proteins in question, they do not appear to resolve the issue of the Illinois-grown controls.

Therefore, the question remains, how did the control samples in this experiment become contaminated and if they were not contaminated, was there expression of the proteins in the control plants. Although the majority of the control samples contained 2% or less of the amount of each protein, compared to the transgenic, two of the control samples contained 8.1 & 9.3% of the amount of protein compared to the transgenic. These numbers are somewhat puzzling and without a more detailed explanation, they are troubling.

As they are presented, the overall numbers do support the suggestion by AgrEvo that the Cry9C and PAT proteins represent a relatively small amount of the total proteins found in the transgenic plants. However, this is based upon the assumption that the titers of the proteins provided in this report are accurate. Because the control samples did show positive signals for each proteins, the accuracy of these numbers is questionable.

CLASSIFICATION: SUPPLEMENTAL. This submission can be upgraded to ACCEPTABLE with submission an adequate explanation for why the control samples also showed positive ELISA results for the PAT and Cry9C proteins, or supplemental data to address this issue.

9414

APPENDIX TO PROTEIN STABILITY

10/9/14

Table 1 Critical Dates for Corn Grains and Processed Fractions

Biotech Sample ID	Matrix	Line	Processed at Texas A&M	Received at ARC	Sample ground	Samples extracted	Bradford Protein Assay	PAT & Cry9C ELISA Assay
114A	WC	Control	Jan. 06, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114B	CG (dry mill)	Control	Jan. 08, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114C	CM (dry mill)	Control	Jan. 08, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114D	Flour (dry mill)	Control	Jan. 08, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114E	HM (dry mill)	Control	Jan. 08, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114F	SEG (dry mill)	Control	Jan. 13, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 3, 98
114G	CO (dry mill)	Control	Jan. 13, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114H	RO (dry mill)	Control	Jan. 14, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114I	SC (wet mill)	Control	Jan. 09, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114J	HM (wet mill)	Control	Jan. 10, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114K	Gluten (wet mill)	Control	Jan. 09, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114L	Starch (wet mill)	Control	Jan. 09, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114M	SEGPC (wet mill)	Control	Jan. 12, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 3, 98
114N	CO (wet mill)	Control	Jan. 13, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114O	RO (wet mill)	Control	Jan. 14, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114P	WC	CBH 351	Jan. 06, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114Q	CG (dry mill)	CBH 351	Jan. 08, 98	Jan. 15, 98	Jan. 19, 98 Jan. 30, 98	Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114R	CM (dry mill)	CBH 351	Jan. 08, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114S	Flour (dry mill)	CBH 351	Jan. 08, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114T	HM (dry mill)	CBH 351	Jan. 08, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114U	SEG (dry mill)	CBH 351	Jan. 13, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 3, 98
114V	CO (dry mill)	CBH 351	Jan. 13, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114W	RO (dry mill)	CBH 351	Jan. 14, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114X	SC (wet mill)	CBH 351	Jan. 09, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114Y	HM (wet mill)	CBH 351	Jan. 10, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114Z	Gluten (wet mill)	CBH 351	Jan. 09, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114AA	Starch (wet mill)	CBH 351	Jan. 09, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114AB	SEGPC (wet mill)	CBH 351	Jan. 12, 98	Jan. 15, 98	Jan. 19, 98	Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114AC	CO (wet mill)	CBH 351	Jan. 13, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98
114AD	RO (wet mill)	CBH 351	Jan. 14, 98	Jan. 15, 98		Feb. 02, 98	Feb. 04, 98	Feb. 2, 98

Table 2 Critical Dates for Validation and Recovery Studies of Processed Fractions of Corn Grains from study BK97B04

Biotech Sample ID	Matrix	Line	Processed at Texas A&M	Received at ARC	Sample ground	Samples extracted	PAT ELISA Assay	Cry9C ELISA Assay
78A	WC	Control	Jan. 06, 98	Jan. 15, 98	Apr. 22, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78B	CG (dry mill)	Control	Jan. 08, 98	Jan. 15, 98	Apr. 22, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78C	CM (dry mill)	Control	Jan. 08, 98	Jan. 15, 98		Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78D	Flour (dry mill)	Control	Jan. 08, 98	Jan. 15, 98		Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78E	HM (dry mill)	Control	Jan. 08, 98	Jan. 15, 98	Apr. 22, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78F	SEG (dry mill)	Control	Jan. 13, 98	Jan. 15, 98	Apr. 22, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78G	CO (dry mill)	Control	Jan. 13, 98	Jan. 15, 98		Apr. 23, 98 Apr?28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78H	RO (dry mill)	Control	Jan. 14, 98	Jan. 15, 98		Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78I	SC (wet mill)	Control	Jan. 09, 98	Jan. 15, 98		Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78J	HM (wet mill)	Control	Jan. 10, 98	Jan. 15, 98	Apr. 22, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78K	Gluten (wet mill)	Control	Jan. 09, 98	Jan. 15, 98		Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78L	Starch (wet mill)	Control	Jan. 09, 98	Jan. 15, 98		Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78M	SEGPC (wet mill)	Control	Jan. 12, 98	Jan. 15, 98		Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78N	CO (wet mill)	Control	Jan. 13, 98	Jan. 15, 98		Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98
78O	RO (wet mill)	Control	Jan. 14, 98	Jan. 15, 98		Apr. 23, 98 Apr. 28, 98 May 4, 98	Apr. 28, 98 May 4, 98	Apr. 23, 98 Apr. 28, 98 May 4, 98

WC: Whole corn
CG: Composite grits
CO: Crude oil
RO: Refined oil

11 of 14

Table 3 Limit of Quantitation (LOQ) in PAT and Cry9C ELISAs of Control Corn Samples

Biotech Sample ID	Matrices	PAT ELISA LOQ[a] (ng/mL)	Cry9C ELISA LOQ[a] (ng/mL)
78A	Whole Corn (RAC)	2.01	0.47
78B	Composite Grits (dry mill)	2.50	0.47
78C	Composite Meal (dry mill)	0.47	0.47
78D	Flour (dry mill)	0.47	0.47
78E	Hull Material (dry mill)	0.47	0.47
78F	Solvent Extracted Germ (dry mill)	6.40	0.47
78G	Crude Oil (dry mill)	0.80	0.47
78H	Refined Oil (dry mill)	0.47	0.47
78I	Steepwater Concentrate (wet mill)	0.82	0.47
78J	Hull Material (wet mill)	0.47	0.47
78K	Gluten (wet mill)	0.47	0.47
78L	Starch (wet mill)	0.47	0.47
78M	Solvent Extracted Germ Press Cake (wet mill)	0.47	0.47
78N	Crude Oil (wet mill)	0.47	0.47
78O	Refined Oil (wet mill)	0.47	0.47

[a] The LOQ was determined as either the LOQ of the assay (lowest standard used), or by calculation from assays of matrix which did not contain PAT or Cry9C, whichever is the higher.

Table 4 Validation of Sample Extraction and PAT ELISA with Fortified Non-transgenic Controls of Corn Samples

		Fortified at 0.9 ng/mL		Fortified at 30 ng/mL	
Biotech Sample ID	Matrices	Detected (ng/mL)	%Recovery[a]	Detected (ng/mL)	% Recovery[a]
78A	Whole Corn (RAC)	ND	NA	39.8 ± 7.86	133 ± 26.2
78B	Composite Grits (dry mill)	ND	NA	37.9 ± 5.34	126 ± 17.8
78C	Composite Meal (dry mill)	0.89 ± 0.65	99.3 ± 71.9	34.1 ± 4.22	114 ± 14.1
78D	Flour (dry mill)	ND	NA	33.0 ± 7.35	110 ± 24.5
78E	Hull Material (dry mill)	0.80 ± 0.45	89.1 ± 49.6	33.8 ± 7.67	113 ± 25.6
78F	Solvent Extracted Germ (dry mill)	ND	NA	49.9 ± 7.41	166 ± 24.8
78G	Crude Oil (dry mill)	ND	NA	27.7 ± 6.07	92.5 ± 20.2
78H	Refined Oil (dry mill)	ND	NA	3.55 ± 2.27	11.8 ± 7.55
78I	Steepwater Concentrate (wet mill)	ND	NA	21.5 ± 2.74	71.5 ± 9.13
78J	Hull Material (wet mill)	ND	NA	31.8 ± 6.17	106 ± 20.6
78K	Gluten (wet mill)	0.74 ± 0.64	82.6 ± 71.0	22.2 ± 6.24	73.9 ± 20.8
78L	Starch (wet mill)	ND	NA	8.16 ± 3.31	27.2 ± 11.0
78M	Solvent Extracted Germ Press Cake (wet mill)	0.68 ± 0.33	75.3 ± 36.2	25.8 ± 6.31	85.9 ± 21.0
78N	Crude Oil (wet mill)	ND	NA	17.3 ± 4.00	57.8 ± 13.3
78O	Refined Oil (wet mill)	ND	NA	ND	NA

[a] The recovery is expressed in average of 8 assay replicates from 4 extract replicates for each PAT-fortified non-transgenic matrix.
ND (Not detectable): Below the limit of quantitation.
NA (Not applicable)

12914

Table 5 Validation of Sample Extraction and Cry9C ELISA with Fortified Non-transgenic Controls of Corn Samples

		Fortified at 0.9 ng/mL		Fortified at 30 ng/mL	
Biotech Sample ID	Matrices	Detected (ng/mL)	Mean ± SD % Recovery[a]	Detected (ng/mL)	Mean ± SD % Recovery[a]
78A	Whole Corn (RAC)	1.01 ± 0.47	113 ± 52.5	26.8 ± 2.45	89.3 ± 8.16
78B	Composite Grits (dry mill)	0.53 ± 0.13	58.8 ± 14.3	24.0 ± 1.73	80.0 ± 5.76
78C	Composite Meal (dry mill)	ND	NA	18.4 ± 7.51	61.3 ± 25.0
78D	Flour (dry mill)	ND	NA	21.6 ± 2.58	71.9 ± 8.61
78E	Hull Material (dry mill)	ND	NA	24.5 ± 2.40	81.7 ± 8.00
78F	Solvent Extracted Germ (dry mill)	1.17 ± 0.37	130 ± 41.3	31.8 ± 7.15	106 ± 23.8
78G	Crude Oil (dry mill)	0.52 ± 0.18	58.2 ± 19.7	24.4 ± 5.14	81.2 ± 17.1
78H	Refined Oil (dry mill)	ND	NA	8.63 ± 2.54	28.8 ± 8.48
78I	Steepwater Concentrate (wet mill)	ND	NA	23.3 ± 4.99	77.8 ± 16.6
78J	Hull Material (wet mill)	ND	NA	26.1 ± 7.68	87.1 ± 25.6
78K	Gluten (wet mill)	ND	NA	23.2 ± 3.20	77.3 ± 10.7
78L	Starch (wet mill)	ND	NA	17.6 ± 4.31	58.6 ± 14.4
78M	Solvent Extracted Germ Press Cake (wet mill)	ND	NA	26.9 ± 1.53	89.8 ± 5.11
78N	Crude Oil (wet mill)	ND	NA	23.8 ± 3.51	79.2 ± 11.7
78O	Refined Oil (wet mill)	ND	NA	6.13 ± 2.01	20.4 ± 6.71

[a] The recovery is expressed in average of 12 assay replicates from 6 extract replicates for each Cry9C-fortified non-transgenic matrix.
ND (Not detectable): Below the limit of quantitation.
NA (Not applicable).

Table 6 PAT, Cry9C and Total Extractable Protein Content in Non-transgenic Corn Samples

Sample ID	Matrices	mg TEP/ g sample	ng PAT/ g sample	ng Cry9C/ g sample
114A	Whole Corn (RAC)	3.33 ± 0.11	ND	ND
114B	Composite Grits (dry mill)	2.44 ± 0.19	188 ± 15	231 ± 60
114C	Composite Meal (dry mill)	1.64 ± 0.06	164 ± 28	297 ± 11
114D	Flour (dry mill)	1.72 ± 0.09	61.7 ± 2.7	274 ± 30
114E	Hull Material (dry mill)	1.17 ± 0.07	200 ± 8	296 ± 24
114F	Solvent Extracted Germ (dry mill)	30.3 ± 2.36	1640 ± 140	1610 ± 100
114G	Crude Oil (dry mill)	ND	ND	ND
114H	Refined Oil (dry mill)	ND	ND	ND
114I	Steepwater Concentrate (wet mill)	1.26 ± 0.05	ND	146 ± 10
114J	Hull Material (wet mill)	0.70 ± 0.00	ND	102 ± 5
114K	Gluten (wet mill)	ND	ND	5.03 ± 0.67
114L	Starch (wet mill)	ND	ND	ND
114M	Solvent Extracted Germ Press Cake (wet mill)	1.33 ± 0.08	7.11 ± 1.96	5.25 ± 2.51
114N	Crude Oil (wet mill)	ND	ND	ND
114O	Refined Oil (wet mill)	ND	ND	ND

TEP (Total Extractable Protein)
ND (Not detectable): Below the limit of quantitation.

13914

Table 7 PAT, Cry9C and Total Extractable Protein Content in Transgenic Corn Samples

Sample ID	Matrices	mg TEP/ g sample	ng PAT/ g sample	ng Cry9C/ g sample
114P	Whole Corn (RAC)	3.88 ± 0.14	14800 ± 900	12300 ± 680
114Q	Composite Grits (dry mill)	2.62 ± 0.26	11300 ± 960	9510 ± 1200
114R	Composite Meal (dry mill)	1.74 ± 0.06	5320 ± 200	10700 ± 700
114S	Flour (dry mill)	1.40 ± 0.08	2160 ± 170	8410 ± 700
114T	Hull Material (dry mill)	0.80 ± 0.02	2360 ± 130	6430 ± 300
114U	Solvent Extracted Germ (dry mill)	21.5 ± 2.20	82200 ± 13900	32600 ± 2800
114V	Crude Oil (dry mill)	ND	ND	ND
114W	Refined Oil (dry mill)	ND	ND	ND
114X	Steepwater Concentrate (wet mill)	1.38 ± 0.08	ND	1800 ± 170
114Y	Hull Material (wet mill)	0.78 ± 0.04	32.9 ± 6.14	3490 ± 440
114Z	Gluten (wet mill)	0.69 ± 0.05	ND	1160 ± 50
114AA	Starch (wet mill)	ND	ND	66.3 ± 5.1
114AB	Solvent Extracted Germ Press Cake (wet mill)	2.91 ± 0.36	1900 ± 10	8800 ± 700
114AC	Crude Oil (wet mill)	ND	ND	ND
114AD	Refined Oil (wet mill)	ND	ND	ND

TEP (Total Extractable Protein)
ND (Not detectable): Below the limit of quantitation.

14914

PC CODE: 006466 DP BARCODE: D252310 MRID 447343-06

DATA EVALUATION REPORT

Reviewer: Zig Vaituzis, Senior Scientist Date: 11/10/99
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511C)

Secondary Review by: Mike Mendelsohn Date: 11/10/99
Microbiologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511C)

STUDY TYPE: The study was conducted to compare the performance of male broiler chickens fed transgenic Bt Cry9C insecticidal, glufosinate resistant corn (CBH351) with a standard commercially available corn hybrid.

EPA GUIDELINES NUMBER: Not applicable. This is not a required study.

CITATION: Leeson, S. (1998) The effect of corn hybrid CBH351 on the growth of male broiler chickens. Department of Animal and poultry Science, Arkell Reserch Farms, University of Guelph, Guelph, Ontario, N1G 2W1 Canada. Lab project Id Number C-2-98. April 20,1998. MRID 447343-06

DP BARCODE: D252310 **CASE**: 290730

PETITION #: 9F05050 **CHEMICAL/BIOL#**: 006466 *Bacillus thuringiensis* subsp. tolworthi Cry9C protein

COMPANY/SPONSOR: (Submitter) AgrEvo USA Company, Little Falls Centre One, 2711 Centerville Rd.,Wilmington, De 199808.

TEST MATERIAL: *Bacillus thuringiensis* subsp. tolworthi Cry9C insecticidal, glufosinate resistant corn (event CBH351)

REVIEW CONCLUSION: This is not a required study and the results are inconclusive. Small differences were seen with the CBH351 test groups as compared to the the non-CBH351 test groups. Increased feed intake during the starter period, an increase in bird weight, and greater breast meat yield were observed. However, it is not possible to make an independent assessment of the significance of the data without an analysis of the bird feed for the presence of Cry9C protein.

RECOMMENDATIONS: None. This is not a required study and the results are inconclusive.

MATERIALS & METHODS: Test birds: Three hundred and sixty commercial strain Ross x Ross male broiler chickens obtained at (one) day of age were weighed and randomly distributed to 2 treatment groups, replicated 6 times, with 30 birds per replicate. The replicates were randomized over 12 floor pens each measuring 1.8 x 2.4 m. and cared for according to the guidelines of the

Canadian Council on Animal Care. The birds were reared on 1 of 2 diets (Bt- and Bt+ corn respectively, supplied by AgrEvo). The birds were fed starter diets to day 17 (57% corn as determined from raw data) , grower diets to day 31 (61% corn as determined from raw data) and finisher diets to day 42 (66% corn as determined from raw data).

Test conditions: the birds were maintained at a brooding temperature of 32° C for 5 days, with the temperature being gradually reduced to 22° C by day 33. The report states that constant lighting was used throughout the study, however, the supplied raw data show light periods diminishing from 23 hrs to 12 hrs at day 6, back to 18 hrs at day 14, then to 23 hrs at day 20 to end of study (day 42). Relative humidity was not reported.

Sampling: Feed intake and body weights were recorded at 17, 31 and 42 days. Mortality was recorded as it occurred.

Observations: The study was conducted from January 20, 1998 thru March 3, 1999. At day 42, eight randomly selected birds from each pen were sacrificed and the abdominal fat pad and the right and left breast meat were removed and weighed. No comments on the condition or appearance of the birds are given.

STATISTICAL METHODS: The data were considered by the T-test appropriate to 2 levels of treatment. Significance was accepted at $P < 0.05$.

REPORTED RESULTS: The 31 and 42 day (and 0-42 day) body weights of the birds fed Bt Cry9C, glufosinate resistant corn diets are significantly greater than the birds fed the control corn diet, and the body weight gain measured between days 17 and 31 is also significantly greater for the test birds. The actual difference in body weights was less than 3%. Feed intake for the Bt corn was significantly higher during the starter period (0-17 day). The reported mortality was 3.89% in the control group and 6.11% in the Bt corn group (not significantly different). Post mortem examination revealed a greater breast meat yield (<5%) for Bt fed birds. The feed intake/body weight ratios and percent mortality were unaffected by the source of corn in the diets. Carcass weight and abdominal fat pad and breast meat yield as a percent of carcass weight were also unaffected by the source of corn in the diet.

STUDY AUTHOR'S CONCLUSIONS: Bt Cry9C, glufosinate resistant corn (CBH351) diets are comparable in feeding value to the non-Bt corn hybrid. Actual body weight and breast meat yield was significantly different (<5% for all characteristics evaluated). Therefore in nutritive value CBH351 corn is at least equivalent to the commercial hybrid and did not adversely effect the feeding and growth of the male broiler population used in this study.

QUALITY ASSURANCE MEASURES: No Quality Assurance statement is included with the study report.

DISCUSSION: This was not a required study. In general the testing was conducted by acceptable procedures, and shows no significant mortality or acute effects from the consumption of Cry9C containing corn. However, analytical measurements were not performed as part of this study to

verify the homogeneity, stability or concentrations of the test substance in the test bird diet. This is a substantial deficiency, since small, but significant, differences were seen between the two test groups. Without the analysis of the bird diet it is not possible to make an independent assessment that the noted effects are due to CBH351 corn on the test birds.

The submitted data do show an effect on the feed intake during the starter period, a delayed effect on bird weight and a greater breast meat yield. A verification of the presence of Cry9C in the test diet is essential to determine which treatment group data are a result of Bt in corn.

It is necessary to have the diet analysis data to assess the significance of the reported results.

ADEQUACY OF STUDY: 1. Validation category: Not useful for avian diet effects assessment.
2. Rationale: The submitter does not claim GLP compliance, no Quality Assurance statement is included with the study report, and the report lacks analysis for the presence of Cry9C in the test diet.

385

PC CODE: 006466 DP BARCODE: D240184 MRID 443843-01

EPA Reviewer: Zig Vaituzis, Ph.D. Date: 4/23/98
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division
Primary Reviewer:
Sylvia S. Talmage, Ph.D., D.A.B.T. Signature: See last page
Secondary Reviewers:
Paul G. Forsyth, Ph.D. Signature: See last page
Robert H. Ross, M.S. Signature: See last page

Thru: Phil Hutton, Chief Date: 5/4/98
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511W)

DATA EVALUATION REPORT

STUDY TYPE: Non-Guideline Study

CITATION: MacIntosh, S.C. (1997) Preparation and characterization of catfish pellets. Plant Genetic Systems (America) Inc., 7200 Hickman Road, Ste. 202, Des Moines, IA. PGS Study No. 96QZM005, September 12, 1997. MRID 443843-01.

DP BARCODE: D240184 **CASE**: 061755
REG./FILE#: 070218-R **CHEMICAL/BIOL#**: 006466 *Bacillus thuringiensis* subsp. *tolworthi* Cry9C protein

COMPANY/SPONSOR: Plant Genetic Systems, N.V., J. Plateaustraat 22, B-9000 Gent, Belgium.

TEST MATERIAL: Catfish pellets processed from corn kernels expressing the Cry9C gene incorporated from *Bacillus thuringiensis* subsp. *tolworthi*

REVIEW CONCLUSION: Based on results of a protein-specific ELISA analysis, no Cry9C protein was detectable in catfish pellets processed from corn kernels containing Cry9C protein.

RECOMMENDATIONS: None

ADEQUACY OF STUDY: Supplementary. This study was not intended to fulfill a guideline requirement.

MATERIALS & METHODS: The information in this study is not subject to GLP guidelines because the data are not necessary for product registration. A descriptive protocol (PGS 96QZM005) for the production of catfish pellets was included in the report. Catfish *Bt* pellets (Lot no. PEL-351-0196) were processed from transgenic corn kernels containing the CBH-351 event in a hemizygous form, i.e., containing the insecticidal Cry9C protein and the marker PAT (phosphinothricin acetyltransferase) which confers tolerance to phosphinothricin, the active ingredient in the herbicide, glufosinate-ammonium. Control material was catfish pellets (Lot no. PEL-351-0196C) processed from nontransgenic corn kernels. Reference material consisted of *Bt* whole corn kernels (Lot no. WCR-351-0196) and control whole corn kernels (Lot no. WCR-351-

0196C). Corn kernels processed to produce the test material were harvested from a plot containing a segregating population of corn (produced from a cross of hemizygous plants with wild type, therefore segregating 1:1 hemizygous to wild type). The nontransgenic plants were removed by treating the field with glufosinate-ammonium herbicide. The nontransgenic corn plants of the same line were harvested from another field.

Protein was extracted from test and control samples by milling in a blender and extracting according to SOP PGS-9H/0. Protein concentration was determined based on the Bradford protein quantification method (SOP PGS-9E/0). Cry9C content was analyzed by ELISA (Enzyme-Linked ImmunoSorbent Assay) according to SOP PGS-9D/2.

REPORTED RESULTS: The amounts of extractable protein from the *Bt* and control catfish pellets were 4.6 and 4.5 mg/g of pellet, respectively. *Bt* whole corn kernels contained 17.2 μg of Cry9C protein/g of kernel. No Cry9C protein could be detected in the *Bt* or control catfish pellets.

DISCUSSION: The study was not conducted in accordance with EPA GLP guidelines but was, according to the author, designed to meet the spirit of the guidelines. A draft protocol was present at the beginning of the study. Sample and reagent labeling, handling, and data entries did not meet guidelines but were either appropriate or corrected when possible.

Although the analysis part of the study was conducted at Plant Genetic Systems N.V. Belgium, no details on the place and time of growth of the corn plants or processing of the kernels into catfish pellets was given. Many procedures/protocols and raw data were not provided. This, however, is not expected to have any bearing on the results reported in the study.

REVIEWER'S COMMENTS:

A. **Test Procedures:** The procedures used are acceptable to the Agency.

B. **Statistical Analysis:** No analysis is needed since there was no detectable amount of toxin in the catfish food.

C. **Discussion/Risk Assessment**: The study is scientifically sound and no difference was observed between the processed and control replicates. Cry9C activity was shown to be destroyed following the extrusion procedure utilized in a typical fish food manufacturing process. Thus little or no Cry9C exposure to cultured fish is expected from Cry9C expressed in cord.

D. **Adequacy of the Study:**

1. Validation Category: Acceptable (non-guideline)

2. Rationale: This study adequately address potential toxicity concerns for cultured fish exposed to a diet containing Cry9C protein expressed in corn.

29

DATA EVALUATION REPORT

CRY9C PROTEIN

STUDY TYPE: NON-GUIDELINE STUDY

Prepared for

Biopesticides and Pollution Prevention Division
Office of Pesticide Programs
U.S. Environmental Protection Agency
Crystal Station I
2800 Jefferson Davis Highway
Arlington, VA 22202

Prepared by

Chemical Hazard Evaluation Group
Toxicology and Risk Analysis Section
Life Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37831

Task Order No. 21

Primary Reviewer:
Sylvia S. Talmage, Ph.D., D.A.B.T.

Signature: Sylvia S. Talmage
Date: 3/30/98

Secondary Reviewers:
Paul G. Forsyth, Ph.D.

Signature: Paul G. Forsyth
Date: 3-30-98

Robert H. Ross, M.S., Group Leader

Signature: R H Ross
Date: 3-31-98

Quality Assurance:
Susan Chang, M.S.

Signature: Susan Chang
Date: 3-31-98

Disclaimer

This Data Evaluation Report may have been altered by the Biopesticides and Pollution Prevention Division subsequent to signing by Oak Ridge National Laboratory personnel.

Oak Ridge National Laboratory, managed by Lockheed Martin Energy Research Corp. for the U.S. Department of Energy under contract number DE-AC05-96OR22464

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

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

APR 30 1998

NOTE

SUBJECT: Upgrading of Acute Oral Study for the Cry9C Protein

TO: Michael Mendelsohn
Regulatory Action Leader
Biopesticides & Pollution Prevention Division (7511W)

FROM: John L. Kough, Ph.D., Biologist
Microbial & Plant-Pesticide Branch
Biopesticides & Pollution Prevention Division (7511W)

ACTION REQUESTED

To confirm the acceptability of the acute oral toxicity study submitted by Plant Genetic Sciences (PGS now part of AgrEvo) to support the registration of the Cry9C protein.

BACKGROUND

PGS submitted an acute oral study utilizing a purified preparation of the Cry9C protein along with other information to support a safety assessment of that protein as expressed in corn. The company analyzed the test substance for the presence of the Cry9C protein to determine the concentration of the protein in the dose administered. Several methods were utilized to determine protein concentration: Bradford analysis, OD_{280}, ELISA and insect bioassay. The initial review indicated a discrepancy between the Cry9C concentrations by the different methods (memorandum from J. Kough to M. Mendelsohn, 6 January 1998). While discrepancies between the different methods is to be expected, the most reasonable method to give an accurate Cry9C concentration in a protein matrix background, the ELISA, gave a lower reading but was not selected to calculate the dose administered. The company chose the OD_{280} method corrected by an SDS-PAGE, the least specific method for Cry9C, to calculate the values of the dose administered. Since this method choice was not adequately rationalized or supported with data, the administered dose was recalculated by the Agency and lowered from 5,525mg/kg to 3,760mg/kg bodyweight.

RECOMMENDATION

With the company's acceptance of the Cry9C recalculation, the acute oral toxicity study (MRID 442581-07) can be upgraded from SUPPLEMENTAL to ACCEPTABLE. It is not clear at the present time what an appropriate limit dose should be in toxicity testing for proteins through the oral route of exposure. There is no indication that there is a significant hazard of oral toxicity that would be not detected by testing the protein substance at 3,760mg/kg rather the 5,525mg/kg bodyweight.

18 / EPP-#PP5G78

CBI Deleted Copy

7PP

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
MRID NO:	447140-01
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 *Bt* 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.

RECEIVED DEC 21 1999

1 97

CBI Deleted Copy

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.*

CBI Deleted Copy

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

2. DNA Insert, Sequence, and Purity - [ ] 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. Pleiotrophic 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

39.

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).

CBI Deleted Copy

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 allergenic 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 ≥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

597

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 as 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,

697

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.

DATA EVALUATION REPORT

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

Secondary Reviewer: Doug Gurian-Sherman, Ph.D., Plant Pathologist, BPPD

STUDY TYPE:	Acute Oral Toxicity (152A-10)
MRID NO:	442581-07
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 OD_{280} 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

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 Na_2CO_3-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 OD_{280} 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 OD_{280} 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 lyophlyzed preparation which was 85% Cry9C by SDS-PAGE and western blot (data not presented). The LC_{50} value was 72.9ng/ml (insect not specified, protocol not submitted) and Bradford protein was 0.89mg/ml. The ELISA (1.0mg/ml) and OD_{280} (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 OD_{280} 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 OD_{280} value which affects the final dose calculation. The historical data should be submitted to bolster using the OD_{280} in lieu of the typical ELISA values.

28E

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 soluable protein or 12 to 225µg Cry9C/gm fresh weight), there is little likelihood any adverse effects will be seen in dietary exposure.

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.

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

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 Na_2CO_3-HCl; 0.01M DDT; 5mM EDTA; 0.1mM PMSF; pH 10) preserved with 0.02% NaN_3 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/cm^2 diet surface. The second assay was done with both Cry9C and Cry1Ab at 50µg/ml and tested at either 100ng/cm^2 or 1250ng/cm^2 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 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/cm^2 diet surface. The second assay was done with both Cry9C and Cry1Ab at 50µg/ml and tested at either 100ng/cm^2 or 1250ng/cm^2 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 LC_{50} 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/cm^2 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.

DATA EVALUATION REPORT

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

Secondary Reviewer: Doug Gurian-Sherman, Ph.D., Plant Pathologist, BPPD

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
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-endotxins 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.

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-endotxins 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*.

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- Amino Acid Sequence Homology
MRID NO:	443844-04
CHEMICAL NO:	006466- *Bacillus thuringiensis* subsp. *tolworthi* Cry9C toxin
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.

23/CAT # PF-8G

DATA EVALUATION REPORT

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

Secondary Reviewer: Doug Gurian-Sherman, Ph.D., Plant Pathologist, BPPD

4PP

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 ≥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

184

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 compatable 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 ≥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 50µl 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 (0.1µg 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 (0.45µm pore size, BAS85, Schleicher & Schüll), then reacted with sera. The SDS-PAGE was a typical 6cm high12.5% 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 250µM 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 450µM BCIP (5-bromo-4-chloro-indolyl-phosphate disodium salt, SIGMA, St. Louis, MO) and 400µM 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 ≥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.