Safety Assessment of the Neomycin Phosphotransferase II (NPTII) Protein

Article *in* Bio/Technolgy · January 1994

DOI: 10.1038/nbt1293-1543 · Source: PubMed

6 authors, including:

Joel E. Ream
Monsanto Company
27 PUBLICATIONS **888** CITATIONS
SEE PROFILE

Bruce Hammond
PRIVATE CONSULTANT
48 PUBLICATIONS **2,625** CITATIONS
SEE PROFILE

Safety Assessment of the Neomycin Phosphotransferase II (NPTII) Protein

Roy L. Fuchs*, Joel E. Ream, Bruce G. Hammond, Mark W. Naylor, Richard M. Leimgruber and Sharon A. Berberich

The Agricultural Group, A Unit of Monsanto Company, 700 Chesterfield Village Parkway, Chesterfield, MO 63198. *Corresponding author.

Two approaches were used to assess the safety of the NPTII protein for human consumption using purified *E. coli* produced NPTII protein that was shown to be chemically and functionally equivalent to the NPTII protein produced in genetically engineered cotton seed, potato tubers and tomato fruit. The NPTII protein was shown, as expected, to degrade rapidly under simulated mammalian digestive conditions. An acute mouse gavage study confirmed that the NPTII protein caused no deleterious effects when administered by gavage at a cumulative target dosage of up to 5000 mg/kg of body weight. This dosage correlates to at least a million fold safety factor relative to the average daily consumption of potato or tomato, assuming all the potatoes or tomatoes consumed contained the NPTII protein. These results, along with previously published information, confirm that ingestion of genetically engineered plants expressing the NPTII protein poses no safety concerns.

Received 8 September 1993; accepted 15 October 1993.

A number of genetically engineered plants have been generated and field tested to assess their agronomic performance and efficacy[1]. Over 400 field tests representing a broad spectrum of plants expressing a wide range of traits have been conducted to date[2]. Many of these plants were generated using the gene encoding neomycin phosphotransferase II (NPTII), also referred to as the amino-glycoside-3′-phosphotransferase II [APH(3′)II], protein as the selectable marker[1]. As these plants progress through field testing and into market introduction, the safety of these plants, plant products and the expressed proteins is being assessed. Because the NPTII protein continues to be used widely as a selectable marker, its safety has received considerable focus. Calgene, Inc. has submitted a petition to the United States Department of Agriculture (USDA) and received deregulated status under the Plant Pest Act[3] for their Flavr Savr® tomato that contains the NPTII protein. Calgene, Inc. has also submitted a petition to the US Food and Drug Administration (FDA) for an advisory opinion[4] and recently converted this submission to a food additive petition[5] addressing the food and feed safety of this marker protein. Nap et al.[6] published an environmental assessment, concluding that the expression of the NPTII protein in genetically engineered plants will not give plants any selective advantage outside the laboratory compared to the non-engineered parental plants. Based on the currently available data on the occurrence and safety of the NPTII protein, Flavell et al.[7] concluded that the marker genes (including the gene encoding NPTII) pose no additional risk to man or the environment.

This report focuses specifically on the food safety of the NPTII protein. Data were generated to address several questions. Does the NPTII protein survive the gastrointestinal system and pose a threat to the therapeutic use of kanamycin and neomycin antibiotics? Does the NPTII protein show unusual resistance to degradation by the proteases in the gastrointestinal system and thus potentially pose a threat as an allergen? Is the NPTII protein itself inherently toxic? These questions were partially addressed previously[4,5]. We employed two primary approaches to address these questions in more detail. The first was to assess the digestive fate of the NPTII protein. Most proteins in food are rapidly degraded upon consumption and exposure to the proteases and acid conditions of the mammalian digestive tract[8]. The fate of the NPTII protein during mammalian digestion was assessed using a simulated, *in vitro* digestion model that has been used widely to investigate the digestibility of plant proteins[9,10], animal proteins[11] and food additives[12]; to assess the protein quality[13]; to study digestion in pigs and poultry[14]; to measure tablet dissolution rates to assess biodegradation of pharmaceutical products[15]; and to investigate the controlled-release properties of experimental pharmaceuticals[16]. Data generated in this study confirm that the NPTII protein is rapidly degraded under these simulated mammalian digestion conditions. These data confirm similar findings submitted to the FDA by Calgene, Inc.[4,5].

The second approach, employed to directly assess the mammalian safety of the NPTII protein expressed in genetically engineered plants, was to administer exaggerated doses of purified NPTII protein by gavage to mice. An acute study was selected since proteins that are toxic to animals produce toxic effects following acute exposure[17,18]. There are no reported instances in which proteins have been shown to be either mutagenic or carcinogenic[18,19]. An exaggerated target dose (5000 mg

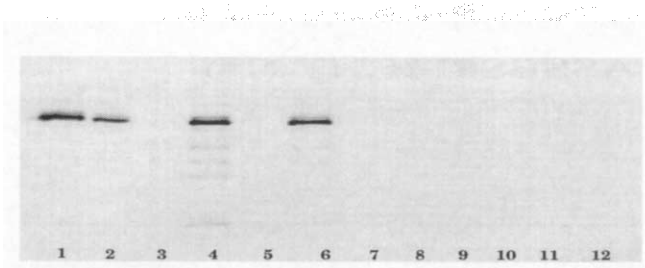

FIGURE 1. Degradation of NPTII protein in simulated gastric fluid as analyzed by western blot. The western blot shown is representative of results from three replicates per treatment. Lanes 1 and 2, NPTII protein standards at 10 (lane 1) and 5 (lane 2) ng/lane; lanes 3 and 4, unincubated buffer without (lane 3) and with (lane 4) addition of NPTII protein; lane 5, unincubated SGF; lanes 6 through 11, samples from NPTII protein incubations for 0 (lane 6), 10 (lane 7), 20 (lane 8), 30 (lane 9), 60 (lane 10) and 120 (lane 11) seconds at 37°C; and lane 12, SGF after 120 seconds incubation at 37°C.

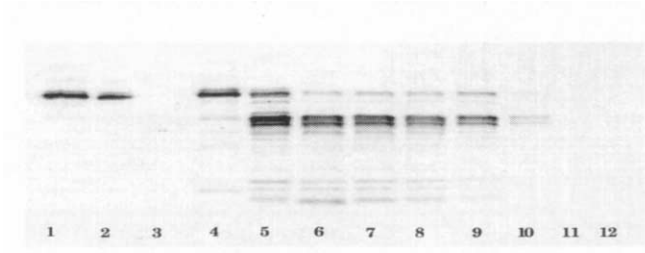

FIGURE 2. Degradation of NPTII protein in simulated intestinal fluid as analyzed by western blot. The western blot shown is representative of results from three replicates per treatment. Lanes 1 and 2, NPTII protein standards at 10 (lane 1) and 5 (lane 2) ng/lane; lanes 3 and 12, SIF after 0 (lane 3) and 15 (lane 12) minutes incubation at 37°C; lane 4, NPTII protein added to unincubated buffer; lanes 5 through 11, samples from NPTII protein incubations for 0 (lane 5), 10 seconds (lane 6), 30 seconds (lane 7), 1 min (lane 8), 2 min (lane 9), 5 min (lane 10) and 15 min (lane 11) at 37°C.

of NPTII protein/kg body weight) was used which corresponds to the Maximum Hazard Dose stated in the Subdivision M guidelines from the US Environmental Protection Agency for biochemical pesticides[20]. Two lower target doses (1000 and 100 mg/kg body weight) were also included. The NPTII protein used for this study was previously shown to be chemically and biologically equivalent to the NTPII protein expressed in genetically engineered cotton seed, potato tubers and tomato fruit[21].

Results

NPTII rapidly degrades in simulated digestive fluids. The susceptibility of the NPTII protein to proteolytic degradation was evaluated in both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) to assess the fate of NPTII under mammalian digestive conditions. NPTII degrades extremely rapidly in SGF. No NPTII protein was detected, by western blot analysis, at the first incubation time point of 10 seconds (Fig. 1). In SIF, NPTII also degrades readily with 50% degradation occurring after 2 to 5 minutes of incubation at 37°C (Fig. 2).

SGF and SIF alone did not produce bands that interfere with western blot analysis for the NPTII protein (Figs. 1 and 2). The recovery of NPTII protein from the digestive fluids was estimated by comparison of protein added to digestive fluid with no incubation to protein added to buffer instead of digestive fluid. Recovery of the NPTII protein was approximately 100% from SGF and approximately 50% from SIF (Figs. 1 and 2). The loss of some NPTII protein in unincubated SIF is likely due to degradation during the assay quench procedure utilized.

A further control was performed to assess whether the 50 μl aliquot removed from the 1 ml incubation solution was representative of the entire incubation solution. It is conceivable that rapid precipitation of an added protein in the digestive fluid could account for lack of NPTII protein in 50 μl aliquots analyzed by western blot. This was addressed by adding protein denaturing buffer to the entire incubation solution and comparing the results from an aliquot of the incubation solution. For the NPTII protein in both SGF and SIF, the western blot results were similar for aliquot and whole-sample treatments. These results established that the results for aliquot samples are representative of the entire incubated digestive fluids.

The disappearance of NPTII protein in SGF and SIF as determined by western blot analysis was consistent with the loss of NPTII functional activity. The enzymatic activity of the NPTII protein was essentially completely destroyed by a 2 minute incubation in SGF and 15 minute incubation in SIF (Table 1). This correlates with the loss of western blot band intensity under similar conditions (Figs. 1 and 2). If earlier time points had been analyzed, the loss of functional activity of the NPTII protein would have been detected sooner.

TABLE 1. Loss of NPTII enzymatic activity in simulated gastric (SGF) and intestinal (SIF) fluids.

	Incubation time (minutes)	NPTII Activity (U/mg)[1]
SGF	0	6.55 (0.11)
	2	0.02 (0.03)
SIF	0	7.67 (0.18)
	15	0.01 (0.05)

[1]One unit (U) is that amount of NPTII activity that catalyzes one μmole of NPTII-dependent NADH consumption per minute at 37°C. NPTII activity values reported are the mean value from three replicate treatments. The standard deviations are in parentheses.

NPTII protein causes no deleterious effects in an acute gavage mouse study. Exaggerated target doses (5000, 1000 and 100 mg/kg body weight) of purified NPTII protein were administered to 10 male and 10 female mice by gavage to further assess the safety of the NPTII protein. A split dose (1 ml/dose), administered in two equal doses, over a 4 hour period was required to deliver the targeted quantity of NPTII protein since the NPTII protein was not soluble at the 150 mg/ml concentration required for a single dose. The estimated concentration and integrity of NPTII protein in the highest dose (before and following the gavage administrations) was confirmed by SDS-PAGE and western blot analysis. The concentration of the high dose was ≥ 75 mg/ml (as calculated) and NPTII protein was shown to be stable in the dosing solutions. Mice were administered the appropriate dose on day 1 and supplied water and diet *ad libitum* for the remainder of the 7 day test. No mortality or moribundity was observed in any of the control or treatment groups during the course of this study. No differences in cage-side behavior or clinical observations were observed. There were no significant differences in group mean terminal body weights (Table 2) and no differences in gross lesions were observed at necropsy compared to the vehicle control.

There were no statistically significant differences in food consumption for males at any of the treatment levels. Food consumption for the female groups for the high dose (5000 mg/kg) and low dose (100 mg/kg) were significantly different ($p \leq 0.05$) from the vehicle control group (Table 3). However, the amount of food "consumed" by the female vehicle control was nearly twice that for any of the male groups. Since

no differences in terminal body weights were observed within sexes or between sexes (Table 2), this difference in "food consumption" probably reflects spillage due to the digging behavior of mice and not differences in actual food consumption. The observed differences in food consumption were, therefore, not attributed to the treatment.

Discussion

It is widely recognized that most proteins rapidly degrade upon consumption and exposure to the mammalian digestive tract[4-16,18,19]. The gastrointestinal tract is specifically designed to digest ingested dietary proteins by conversion to amino acids and small peptides, which are absorbed by the intestinal tract. Hundreds of thousands of proteins are consumed daily in the human diet. Typical eukaryotic cells, including cells comprising food products derived from plants and animals, contain 5000 or more different polypeptides[22], which must be degraded in the digestive system to produce the amino acids required for growth. Demonstration of the rapid degradation of the NPTII protein under simulated digestive conditions confirmed that the NPTII protein is rapidly digested, as expected for other dietary proteins and as was previously reported by Calgene, Inc.[4,5].

The enzymatic activity of NPTII, phosphorylation of kanamycin or neomycin, requires the cofactor ATP. ATP is unstable in the low pH of the digestive system and endogenous concentrations in the stomach are below that required for catalytic activity[6]. These data, along with previous assessments for NPTII protein[4-7] support the conclusion that ingestion of plant products containing the NPTII protein will not compromise the efficacy of the kanamycin or neomycin antibiotics taken orally. Furthermore, the limited use of these antibiotics for humans have been replaced by more effective aminoglycoside antibiotics (e.g., amikacin and netilmycin) that are not substrates for the NPTII protein[6].

Rapid degradation of the NPTII protein by the simulated digestive system also suggests that the consumption of genetically engineered food products expressing the NPTII protein should not pose any significant allergenic concerns. Several reports[23,24] have concluded that one of the critical characteristics of the major allergenic food proteins is their resistance to proteolytic degradation during digestion. For a food protein to elicit an allergenic response, the protein must survive the acid and proteolytic environment of the gastrointestinal system to reach and be absorbed through the intestinal mucosa and trigger an IgE-mediated series of responses. The very rapid degradation rate of the NPTII protein (loss of detectable protein in < 10 seconds) in SGF as measured by both western blot analysis and enzymatic activity strongly suggests that this protein will be readily degraded in the stomach when ingested by mammals. To put the rapid degradation of the NPTII protein in the simulated gastric model into perspective, solid food has been measured, using isotopically-labeled meal, to empty from the human stomach by about 50% in two hours, while liquid empties 50% in approximately 25 minutes[25]. The NPTII protein should not survive the gastric system and, therefore, not be present in the intestine to elicit the IgE-mediated series of responses. If some portion of the NPTII protein did survive the gastric system, it would rapidly be degraded in the intestine. Greater than 50% of the NPTII protein was shown to degrade in the model intestinal fluid in 2 to 5 minutes. This compares with transit times through the intestine, using isotopically-labeled chromate (which is not absorbed in the intestine), of 4 to 10 hours for the first to appear in the feces and 68 to 165 hours for the last to be detected[26]. So, there will be extremely minimal, if any, potential for the NPTII protein to reach the intestinal mucosa to trigger an IgE-mediated response.

Kanamycin resistance, a portion of which is conferred by the *nptII* gene, is also common in bacteria, including bacteria that colonize the gastrointestinal system[4-7]. From the prevalence of bacteria that are resistant to kanamycin, Nap et al.[6] concluded that a continuous exposure to the NPTII protein can be assumed without concern raised as to its safety. Long termed exposure also supports the lack of allergenicity concerns. Furthermore, Calgene, Inc.[4,5] showed that the NPTII protein shows no homology to any of the known allergenic proteins contained in several computer data bases. We have updated these analyses to more recent data bases, again finding no homology to any reported allergen (unpublished).

To address the question of the potential toxicity of the NPTII protein itself, mice were fed exaggerated doses of the NPTII protein by acute gavage. An acute study was selected since proteins known to be toxic to mammals manifest toxicity in a acute exposure[17,18]. The NPTII protein, purified from *E. coli* and shown to be chemically and biologically equivalent to the NPTII protein produced in cotton seed, potato tubers and tomato fruit[21], was fed to mice at the highest targeted dose of 5000 mg/kg body weight. The exaggerated dose (Maximum Hazard Dose) is consistent with the EPA Subdivision M Guidelines for biochemical pesticides[20]. Two one ml doses (2500 mg/kg body weight each) were administered by gavage approximately four hours apart, as a result of the inability to dissolve sufficient NPTII protein for a single dose. As expected, no treatment related mortality or moribundity, differences in weight gain, food consumption, behavior or clinical signs were observed. There were no grossly

TABLE 2. Summary of terminal body weights of mice administered NPTII protein or the vehicle control.

Test Group[1]	Sex	Mean Body Weight[2] (gm)
Vehicle control	Male	28.42 (1.60)
100 mg/kg NPTII	Male	28.40 (1.49)
1000 mg/kg NPTII	Male	28.31 (1.13)
5000 mg/kg NPTII	Male	28.51 (1.73)
Vehicle control	Female	22.93 (1.60)
100 mg/kg NPTII	Female	22.17 (1.02)
1000 mg/kg NPTII	Female	22.00 (0.73)
5000 mg/kg NPTII	Female	21.98 (1.55)

[1]Each test group consisted of 10 randomized mice.
[2]Numbers in parentheses are the standard deviations.

TABLE 3. Summary of food consumption of mice administered NPTII protein or the vehicle control.

Test Group[1]	Sex	Mean Food Consumption[2] (gm)
Vehicle control	Male	4.9 (0.50)BT
100 mg/kg NPTII	Male	4.7 (0.51)*
1000 mg/kg NPTII	Male	5.4 (1.15)
5000 mg/kg NPTII	Male	5.2 (1.35)
Vehicle control	Female	9.4 (3.10)
100 mg/kg NPTII	Female	5.8 (1.94)*
1000 mg/kg NPTII	Female	7.5 (3.11)
5000 mg/kg NPTII	Female	6.1 (2.40)*

[1]Each test group consisted of 10 randomized mice, except for the male 100 mg/kg, the female vehicle control and 5000 mg/kg treatments, which consisted of 9 observations (the remaining treatments were not included due to excessive diet spillage, etc.).
[2]Numbers in parentheses are the standard deviations.
*denotes a statistically significant difference ($p < 0.05$) from the vehicle control using Dunnett's Two-Tailed Test[30]. BT denotes statistically significant difference among variances of the different groups ($p \leq 0.01$) using Bartlett's Test[31].

observable pathologic changes in mice subjected to a comprehensive post-mortem examination. This included internal examination of body cavities and organs *in situ*, opening and examination of hollow organs, and opening and examination of the entire gastrointestinal tract. The 5000 mg/kg body weight dose for NPTII protein used for this study would be equivalent to an average human consuming, in one day, more than one million tomato fruit or potato tubers expressing the level of NPTII protein previously reported for these genetically engineered crops[21,27].

Whole food feeding studies for a longer duration (28 days) with genetically engineered tomato fruit, potato tubers and raw, ground cotton seed that express the NPTII protein also showed no adverse consequences (unpublished data). Rats were fed potatoes or tomatoes at a level equivalent to an average human consuming 40 raw potatoes or 100 tomatoes per day for 28 days with no adverse effects. These data were generated with lower doses of NPTII protein than used in the mouse acute gavage study, but with exaggerated food consumption levels compared to the average daily human consumption of these foods[28].

In sum, these studies, which are consistent with those recommended previously[29,30] demonstrate that the NPTII protein is readily degraded like other dietary proteins, will not compromise the efficacy of aminoglycoside antibiotics, does not possess the attributes of known protein food allergens, is not toxic to mammals and, hence, presents no risks for human or animal consumption. Combined with the information published by Calgene, Inc.[4,5], Nap et al.[6] and Flavell el al.[7] these data establish that the NPTII protein produced in genetically engineered plants poses no discernable environmental, food or feed safety concerns.

Experimental Protocol

Digestive fluids for metabolic fate studies. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described in the United States Pharmacopeia[31]. Solutions were stored at 4°C and used the day they were prepared. SIF was mixed well before use to resuspend any insoluble material that settled after preparation. Digestive fluids were assayed for proteolytic activity prior to use in digestion assays. Pepsin activity was measured by monitoring the increase in absorbance at 280 nm following trichloroacetic acid precipitation of SGF digestion of hemoglobin[32]. Protease activity of SIF was assayed by measuring, spectrophotometrically, the increase in supernatant A_{574} following trichloroacetic acid precipitation of SIF incubations with resorufin labeled casein as described by the manufacturer (Boehringer Mannheim Corporation, Indianapolis, IN).

Digestion with simulated gastric and intestinal fluids. NPTII protein was added to 1 ml solutions of temperature-equilibrated SIF or SGF to a final concentration of 2 μg/ml and incubation at 37°C. Digestion solutions were agitated as much as practical. Very short incubation experiments (≤2 minutes) were not agitated. Agitation was stopped periodically to remove aliquots. Fifty μl aliquots were removed from the digestion solutions at specific intervals and the reaction was immediately quenched. For incubations in SGF the reaction was quenched by neutralization with 15 μl of 0.2 M sodium carbonate per 50 μl of SGF. For samples to be analyzed by western blot, quenched aliquots were kept on an ice bath until dilution (1:1, v/v) with 2X sample buffer [100 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, and 0.2% (w/v) bromophenol blue][33]. Samples were heated for five minutes at 100°C and stored at approximately -20°C until analysis. For incubations in SIF in which the analysis was going to be by western blot, the reaction was terminated by immediate 1:1 (v/v) dilution with 2X sample buffer, followed by immediate incubation at 100°C for five minutes. These samples were stored on an ice-bath until all incubations were complete and then transferred to -20°C until analysis. For samples measuring protein levels with no incubation in digestion fluids (*i.e.* t=0), protein was added to SGF and SIF after addition of quenching reagent. To allow a calculation of the recovery of NPTII protein from digestion fluid, NPTII was added to 1 ml solutions of buffer and then sampled and quenched as before. An additional control was prepared to allow the determination if the 50 μl aliquot was representative of the entire digestion incubation solution. For this sample, the digestion incubation volume was reduced to 0.1 ml and the entire sample was quenched.

Western blotting. Samples in 1X sample buffer[33] were thawed and reheated at 100°C, allowed to cool, and subjected to SDS-PAGE[33] and western blotting[34]. Rabbit antibody raised against native NPTII was used for western blot analyses. Protein recoveries and the extents of degradation were estimated based on visual comparison of relative intensities of bands corresponding to standards for three replicates for each treatment. When intermediate levels of protein were detected with increasing incubation time, the estimated level relative to initial levels were averaged and the extent of degradation estimated. For some treatments, protein degradation was too fast or too slow to observe intermediate levels; for these, the limits of degradation were estimated.

NPTII enzyme activity. NPTII activity was measured before and after incubation in digestive fluids. NPTII was added to SGF and SIF to a final concentration of 10 μg/ml and incubated as described previously. SGF incubations were quenched by the addition of 0.3 ml of 0.2 M sodium carbonate to the 1 ml incubation solution. An unincubated control was prepared by adding the NPTII to SGF after the addition of the quench solution. SIF incubations were quenched by immediate placement of the incubation sample on an ice bath. Incubation solutions were assayed for NPTII enzymatic activity immediately after incubation by monitoring the neomycin-dependent change in absorbance at 340 nm using the continuous coupled spectrophotometric assay described by Goldman and Northrop[35].

Dose formulation of NPTII protein. The NPTII protein produced in *E. coli*, purified, characterized and shown to be chemically and functionally equivalent to the NPTII protein expressed in genetically engineered cotton, potato and tomato, was produced as a lyophilized powder as described[21]. For the metabolic fate study, lyophilized NPTII protein was dissolved in 50 mM Tris-HCl (pH 7.8) buffer containing 20% (w/v) glycerol and 1 mM β-mercaptoethanol at the concentrations stated for these studies. For the mouse acute gavage study, lyophilized NPTII protein was dissolved in 50 mM sodium carbonate buffer (pH 9.6) to produce NPTII concentrations of 75, 15 and 1.5 mg/ml. These concentrations were sufficient to enable administration of 2, 1 ml doses to mice and achieve doses equivalent to 5000, 1000 and 100 mg NPTII/kg mouse body weight, assuming a mouse weight of 30 g. The vehicle control consisted of 50 mM sodium carbonate buffer (pH 9.6).

Dose confirmation and integrity. The concentration and integrity of NPTII in the highest concentration (75 mg/ml) administered to mice was confirmed by SDS-PAGE[33] and western blot analysis[34]. Aliquots of the NPTII dosing preparation were sampled prior to and following the gavage experiment and stored at -80°C until analyzed. Samples were thawed, diluted with SDS-PAGE buffer and analyzed by both methods.

Acute gavage study. Albino mouse, strain CD-1, were obtained from the Charles River Breeding Laboratory, Portage, MI and acclimatized for 14 days. Ten mice were randomly assigned to the eight treatment groups (0, 100, 1000 and 5000 mg of NPTII/kg body weight) for males and females. Males (ranging from 30.3 to 37.3 grams) were approximately seven weeks old at the start of the study and the females (ranging from 23.3 to 30.8 grams) were nine weeks old. Mice were individually ear tagged for identification. After gavage administration of NPTII protein, mice were supplied with water and food (Purina Certified Rodent Chow, number 5002) *ad libitum*. A 12 hour light cycle was used daily. Mice were administered the designated amount of NPTII protein (or the vehicle control) by gavage. The total dose was divided into two equal amounts, due to the limit of solubility of the NPTII protein (approximately 100 gm/ml), which were given approximately four hours apart on day one of the study. Ten mice per sex per dose were given NPTII protein at the target doses of 0, 100, 1000 and 5000 mg/kg of body weight. Control mice were administered only the vehicle at 1 ml per gavage time point.

Clinical observations. Clinical observations, mortality and moribundity, were assessed twice daily. Detailed observations for signs of toxicity were performed on day 7. Body weights were determined prior to randomization and on day 7. Food consumption was measured on days 1 and 7. The Dunnett's Multiple Comparison Test (two-tailed)[36] and Bartlett' Test[37] were used to detect statistically significant differences between treated animals and the respective control. Terminal body weights were determined and reported with appropriate standard deviations. All males were sacrificed on day 8 and females on day 9. All animals were necropsied. Internal cavities were opened and organs examined *in situ*, then removed. Hollow organs were opened and examined.

Reagents. Pepsin (porcine), pancreatin (porcine), hemoglobin (bovine), lactate dehydrogenase (rabbit muscle), pyruvate kinase (rabbit muscle) and neomycin sulfate were obtained from Sigma Chemical Company (St. Louis, MO). Resorufin-labeled casein was obtained from Boehringer Mannheim Corporation (Indianapolis, IN). All other reagents were reagent grade obtained from commercial sources.

Acknowledgments

We thank Steve Rogers, David Bartnicki, Paul Lavrik and Terry Stone for their critical review of this manuscript and Glen Rogan for his excellent technical contributions.

References

1. Gasser, C. S. and Fraley, R. T. 1989. Genetically engineering plants for crop improvement. Science **244:**1293–1299.
2. Casper, R. and Landsman, J. 1992. Summary of results, p. 12–14. *In:* The Biosafety Results Of Field Tests Of Genetically Modified Plants And Microorganisms. Casper, R. and Landsmann, J., Biologische Bundesanstalt fur Land- und Forstwirtschaft, Braunschweig, Germany.
3. Animal and Plant Health Inspection Service. 1992. Proposed interpretive ruling in connection with Calgene, Inc. petition for determination of regulatory status

of Flavr Savr® Tomato. Fed. Reg. **57:**31170.
4. Calgene, Inc. 1990. Request for advisory opinion Kan^R gene: safety and use in the production of genetically engineered plants. FDA Docket Number: **90A-0416.**
5. Calgene, Inc. 1993. Food additive petition for the APH(3′)II as a processing aid. FDA docket number: **93F-0232.**
6. Nap, J. P., Bijvoet, J. and Stikema, W. J. 1992. Biosafety of kanamycin-resistant transgenic plants: an overview. Transgenic Crops **1:**239–249.
7. Flavell, R. B., Dart, E., Fuchs, R. L. and Fraley, R. T. 1992. Selectable marker genes: safe for plants? Bio/Technology **10:**141–144.
8. Guyton, A. C. 1981. Digestion and absorption in the gastrointestinal tract, p. 816–826. *In:* Textbook of Medicinal Physiology, 6th Ed. W. B. Saunders Co., Philadelphia.
9. Nielson, S. S. 1988. Degradation of bean proteins by endogenous and exogenous proteases—a review. Cereal Chem. **65:**435–442.
10. Marquez, U. M. L. and Lajolo, F. M. 1981. Composition and digestibility of albumin, globulins, and glutelins from *Phaseolus vulgaris*. J. Agric. Food Chem. **29:**1068–1074.
11. Zikakis, J. P., Rzucidlo, S. J. and Biasotto, N. O. 1977. Persistence of bovine milk xanthine oxidase activity. J. Dairy Science **60:**533–541.
12. Tilch, C. and Elias, P. S. 1984. Investigation of the mutagenicity of ethylphenylglycidate. Mutation Research **138:**1–8.
13. Akeson, W. R. and Stahmann, M. A. 1964. A pepsin pancreatin digest index of protein quality evaluation. J. Nutrition **83:**257–261.
14. Fuller, M. F. (Ed.). 1991. *In Vitro* Digestion for Pigs and Poultry. C.A.B. International, Wallingford, UK.
15. Alam, A. S., Hagerman, L. M. and Imondi, A. R. 1980. Bioavailability of sulpiride tablet and capsule in dogs. Arch. Int. Pharmodyn. Ther. **247:**180–189.
16. Doherty, A. M., Kaltenbronn, J. S., Hudspeth, J. P., Repine, J. T., Roark, W. H., Sircar, I., Tinney, F. J., Connolly, C. J., Hodges, J. C., Taylor, M. D., Batley, B. L., Ryan, M. J., Essenburg, A. D., Rapundalo, S. T., Weishaar, R. E., Humblet, C. and Lumney, E. A. 1991. New inhibitors of human renin that contain novel replacements at the P_2 site. J. Med. Chem. **34:**1258–1271.
17. Li, A. P. 1989. Protein toxicology applied to biotechnology products. *In:* Genetically Engineered Plants: Scientific Issues In Their Regulation For Animal Feed And Human Food Uses, Workshop at Boyce Thompson Institute for Plant Research and Cornell University, Ithaca, New York.
18. Jones, D. D. and Maryanski, J. H. 1991. Safety considerations in the evaluation of transgenic plants for human food, p. 64–82. *In:* Risk Assessment In Genetically Engineering: Environmental Release Of Organisms. Levin, M. and Strauss, H. (Eds.). McGraw-Hill, New York.
19. Pariza, M. W. and Foster, E. M. 1983. Determining the safety of enzymes used in food processing. J. Food Protection **46:**453–468.
20. Pesticide testing guidelines. Subdivision M. Microbial and Biochemical Pest Control Agents. PB89-211676. National Technical Information Service, Springfield, VA.
21. Fuchs, R. L., Heeren, R. A., Gustafson, M. E., Rogan, G. J., Bartnicki, D. E., Leimgruber, R. M., Finn, R. F., Hershman, A. and Berberich, S. A. 1993. Purification and characterization of microbially expressed neomycin phosphotransferase II (NPTII) protein and its equivalence to the plant expressed protein. Bio/Technology **11:***This issue*.
22. Kessler, D. A., Taylor, M. R., Maryanski, J. H., Flamm, E. L. and Kahl, L. S. 1992. The safety of foods developed by biotechnology. Science **256:**1747–1749.
23. Taylor, S. L., Nordlee, J. A. and Bush, R. K. 1992. Food allergies, p. 316–329. *In:* Food Safety Assessment, Ginley, J. W., Robinson, S. F. and Armstrong, D. J. (Eds.). ACS Symposium Series 484. ACS, Washington, DC.
24. Taylor, S. L., Lemanske, R. F. Jr., Bush, R. K. and Busse, W. W. 1987. Food allergens: structure and immunologic properties. Ann. Allergy **59:**93–99.
25. Sleisenger, M. H. and Fordtran, M. D. 1989. Gastrointestinal Disease, Volume 1. *In:* Pathophysiology Diagnosis Management, 4th Ed. W. B. Saunders Company, Philadelphia.
26. Davenport, H. W. 1971. Physiology of the Digestive Tract, 3rd Ed. Year Book Medical Publishers, Inc., Chicago.
27. Fuchs, R. L., Stone, T. B. and Lavrik, P. B. 1993. Risk assessment: a technical perspective. *In:* NABC Report 5, Agricultural Biotechnology: A Public Conversation About Risk, J. Fessenden MacDonald (Ed.). National Agricultural Biotechnology Council. Ithaca, NY.
28. United States Environmental Protection Agency (USEPA). 1984. Dietary residue evaluation system (DRES) (formerly TAS: Tolerance assessment system). Washington, D.C.
29. Food and Drug Administration, Department of Health and Human Services. 1992. Statement of Policy: Foods Derived from New Plant Varieties. Fed. Reg. **57:**22984–23005.
30. International Food Biotechnology Council. 1990. Biotechnologies and food: assuring the safety of foods produced by genetic modification. Reg. Toxicol. Pharmacol. **12:**S1–S196.
31. The United States Pharmacopeia, Vol. XXII, p. 1788–1789. United States Pharmacopeial Convention, Inc. Rockville, MD.
32. Ryle, A. P. 1984. Pepsins, gastricsins and their zymogens, p. 223–238. *In:* Methods of Enzymatic Analysis, Vol. V. Bergmeyer, H. U. (Ed.). Verlag Chemie, Weinheim.
33. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature **227:**680–685.
34. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electro-blotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035–10038.
35. Goldman, P. R. and Northrop, D. B. 1976. Purification and spectrophotometric assay of neomycin phosphotransferase II. Biochem. Biophys. Res. Comm. **69:**230–236.
36. Dunnett, C. W. 1955. A multiple comparison procedure for comparing several treatments with a control. J. Am. Stat. Assoc. **50:**10967–1121.
37. Dixon, W. J. and Massey, F. J. Jr. 1969. Introduction to Statistical Analysis, 3rd Ed. McGraw-Hill Co., NY.