

US EPA ARCHIVE DOCUMENT

**BACKGROUND DOCUMENT**  
**FOR**  
**THE FIFRA SCIENTIFIC ADVISORY PANEL**  
**ON**  
**MAMMALIAN TOXICITY ASSESSMENT GUIDANCE FOR**  
**PROTEIN PLANT-PESTICIDES**

June 7, 2000

## INTRODUCTION

In recent years, the application of biotechnology to the development of new products, pharmaceuticals and improved crops has rapidly progressed to the marketplace. Engineered enzymes have already been commercialized and are widely used in, e.g., laundry detergents, cheese production and heart attack medications. In the agricultural arena, several engineered crops have reached the market and been widely grown. Some of these crops have been engineered to protect the crops from damage by insects and disease. Besides the obvious benefits of increased yields in the presence of pests, the use of some of these crops has resulted in reduced use of chemical pest control agents and lowered the level of mycotoxin contamination in the harvested food crop. There has been significant public scrutiny of these products of the new technology, especially overseas, with regards to the robustness of the safety assessments done prior to commercial release. The approval process of engineered plants in the United States involves three different agencies of the federal government: the United States Department of Agriculture's Animal and Plant Health Inspection Service (APHIS), the Food and Drug Administration's Center for Food Safety and Applied Nutrition (FDA-CFSAN) and the Environmental Protection Agency's Office of Pesticide Programs (EPA-OPP).

APHIS is responsible under the Federal Plant Pest Act and Plant Quarantine Act to prevent the introduction and dissemination of plant pests into or within the United States. For experimental trials in the environment, APHIS ensures experiments are done such that there is a low probability of the new trait escaping into other crops or wild plant relatives. When APHIS determines that there are no plant pest risks and that no harm will result to agriculture, the new plant may be grown commercially without restriction, provided no other laws cover the traits. At this point or earlier, developers of food plants consult with FDA about the safety of their product. For plants with altered pest resistance traits, the companies must obtain a plant-pesticide registration from EPA.

FDA is responsible under the Federal Food Drug and Cosmetic Act (FFDCA) for determining the safety of any new food or food ingredients, except for pesticides, which are examined by EPA. Unless a specific safety determination is deemed necessary or requested by the plant developer, the FDA relies on a consultation process whereby the individual plant developer presents nutritional and safety data for the food derived from the plant with new traits. The emphasis of the assessment is on the whole food, not simply the introduced trait, so foods resulting from plant-pesticide expressing crops are also examined by FDA. The food derived from the new plant must not be substantially changed with respect to nutritional composition, requirements for storage, preparation or cooking nor can it contain a new allergen. Developers must consider levels of toxicants known to occur in the plant and any pleiotropic effects (unintended effects) that may have occurred due to the genetic manipulation. FDA also ensures that any label that may be associated with the food product is truthful and not misleading.

Under the Federal Fungicide, Insecticide and Rodenticide Act (FIFRA), EPA is responsible for determining that the plant-pesticide can be used safely in the environment. EPA examines the

plant-pesticide under FIFRA to determine if any unreasonable risks to man and the environment can occur including risks for non-target species and non-dietary exposures for humans. Under the FFDCA, EPA is specifically given the responsibility to determine maximum allowable levels of pesticide residues occurring on foods, termed food tolerances. For assessing the dietary risks of pesticides, EPA examines the toxicity of the pesticidal substance itself. When the plant-pesticide's aggregate exposure has been deemed to possess a reasonable certainty of causing no harm, EPA can determine the maximum allowable dietary exposure and establish a food tolerance. If the data to support a tolerance determination indicates a lack of toxicity, then EPA can grant an exemption from the requirement for a food tolerance. For the plant-pesticides examined to date, the lack of toxicity in the data submitted has justified granting an exemption from the requirement of a food tolerance.

Throughout the registration process, EPA has been aware of new issues that have arisen related to genetically engineered plants and has performed all its science assessments for registration in a transparent manner receiving input both from the public and scientific experts for the pesticidal products currently in the marketplace. This public review and comment includes publishing relevant risk assessment information in the Federal Register and convening Scientific Advisory Panels (SAP). EPA has held public fora to discuss its initial plant-pesticide registrations, pest insect resistance to the *Bacillus thuringiensis* toxins expressed in plants and aspects of food safety including food allergy (<http://www.epa.gov/scipoly/sap/index.htm>). EPA has also recently responded to a petition by Greenpeace International, *et al.*, to revoke all the existing registrations for plant-pesticides utilizing proteins from *Bacillus thuringiensis*. The petition response thoroughly summarizes all the data examined to date by EPA to justify these registrations, as well as published studies addressing the relevant issues, and is available on line (<http://www.epa.gov/pesticides/biopesticides/petition.pdf>). Finally, the National Research Council of the National Academy of Sciences has recently released a report entitled "Genetically Modified Pest-protected Plants, Science and Regulation" that addresses many issues surrounding the plant-pesticides which are regulated by EPA (<http://www.nap.edu/html/gmpp/>). The report also provides a background to the risk issues related to enhanced pest resistance developed by both traditional breeding and genetic modification.

The FIFRA Science Advisory Panel is being convened to obtain the current scientific opinion on methods to assess the mammalian toxicity risks associated with genetically modified plants expressing protein plant-pesticides.

### PROTEIN PLANT-PESTICIDE ISSUES

Traits introduced into plants for the purpose of pest control are pesticides according to the Federal Insecticide Fungicide and Rodenticide Act (FIFRA) and may require a food safety determination under the Food Drug and Cosmetic Act (FFDCA). The Agency calls this special class of pesticides "Plant-Pesticides" and has defined them as the pesticidal substance expressed in the living plant and the genetic material necessary for its expression. The nature of these pest control agents is such that they have a very different risk scenario from traditional synthetic

chemical pesticides. All pesticides, including these protein plant-pesticides, are intended to control pests, often by killing or otherwise discouraging the pest in question. Therefore, these protein pesticidal substances are already known to adversely affect some species. However, unlike chemical pesticides, proteins have a predictable fate in the diet. Proteins are the building blocks of cell biology and their synthesis from the twenty common amino acids is an essential motif for all forms of life. Living organisms have the ability to either synthesize their own amino acids or obtain them from other sources, such as dietary proteins. Animal species are able to degrade proteins found in their diet to peptides and amino acids which they absorb, then incorporate into their own proteins. This essential feature, the typical fate of dietary proteins, is the basis of EPA's approach to the assessment of protein plant-pesticides that require a food tolerance determination under FFDCA.

While providing the structural framework and chemical tools for life, proteins in the form of toxins have also been associated with serious health effects in mammalian species. Bacterial toxins, such as botulinum toxin, tetanus toxin and diphtheria toxin, have historically caused considerable human fatalities before the advent of improved food processing and vaccinations. Plant toxins, such as ricin and abrin, and animal toxins, such as snake and arthropod venoms, are among the most potent poisons known. However, these protein toxins are exceptional cases. The vast majority of proteins in the diet, including a large number of uncharacterised proteins of both plant and animal origin, serve as sources of amino acids essential for proper nutrition and health. Besides toxicity, another hazard with dietary proteins relates to the potential of ingested proteins to become food allergens. EPA recently held another SAP on food allergy (February 29, 2000) and is not asking the present panel to consider the allergenicity issue at this time.

Given that proteins expressed in plants as plant-pesticides are intended to control certain organisms and that EPA has regulatory responsibilities to determine that there is a reasonable certainty that no harm will result from exposure to these proteins, the Agency has developed guidance for persons wishing to register these proteins. The data categories relating to mammalian toxicity and dietary safety supporting the protein plant-pesticides to date are:

- Characterization of the protein expressed and its mode of action
- Amino acid sequence homology analysis for similarities to known toxins and allergens
- Test substance equivalence between plant and microbially produced protein
- In vitro* digestibility assay in simulated gastric and/or intestinal fluids
- Acute oral toxicity test with pure protein plant-pesticide (OPPTS Harmonized Guideline number 870.110)

The guidance follows the rationale for mammalian toxicity outlined for other biopesticides with some modifications due to assumptions about the potential for human exposure. In order to formalize the Agency's approach for establishing the mammalian safety of protein plant-pesticides, the Agency is convening the FIFRA Science Advisory Panel.

#### AMINO ACID SEQUENCE HOMOLOGY

Fortunately, most proteins do not display any toxicity. Most enzymes and structural proteins fall into this grouping (Pariza and Foster, 1983). In addition, those proteins that are known to be mammalian toxins have been well studied (e.g., for bacterial toxins: D.M.Gill, 1987 & C.K. Schmitt *et al.*, 1999 and for toxins in general, The Handbook of Natural Toxins, Vol. 1-8, 1995, ed. by A.T.Tu ). Their amino acid (AA) sequence, if not higher levels of structural organization, have been elucidated. The Agency believes this AA sequence information can be useful as part of a preliminary screen for determining structural relatedness to known protein toxins and allergens. The rationale is that, at some level, AA sequence homology can indicate a close functional relationship between two proteins. To date this has been used to elucidate evolutionary links among different species by examining the AA sequences of functionally identical proteins (e.g., cytochrome C or hemoglobin molecules). However, the homology analysis may suggest functional relationships, since this technique has been used to discover the ubiquity of a developmental trigger shared among species (i.e., the HOX box) and the presence of a common stress protein in many species (i.e., heat shock protein).

In the case of toxicity, the AA sequence homology may be useful to screen for potential protein toxicity in mammals, and indicate the direction of further testing if toxicity is seen in the acute toxicity test. However, there are four problems with the AA sequence homology screening technique. First, there is no existing method to link AA homology directly with protein function. A high sequence homology result simply suggests that the protein in question may exhibit the function indicated, but confirmatory testing would be necessary. Second, there is not, currently, an acceptable level of amino acid homology to trigger closer examination of the introduced protein suspected to be related to a toxin. A third problem is that homology determinations need to identify a weighting function for levels of homology considering protein size (e.g., 90% homology for a 60kD protein is not as similar as 90% homology for a 10kD protein). Fourth, identified areas of high homology can be deceptive for inferences about protein function. Homologies in the structural regions of the protein may not have as much significance for protein function as homologies in sites more closely correlated with the protein activity. This has been observed in the CryI series of  $\alpha$ -endotoxins from *Bacillus thuringiensis* where there are high levels of homology in the section of the protoxin that is cleaved off to render an activated toxin. If this highly homologous cleaved section of the protoxin is not included in a sequence analysis, the level of homology among the active toxins drops significantly.

#### IN VITRO DIGESTIBILITY

One of the assumptions about proteins in the diet is that they are broken down into smaller chains of amino acids, called peptides, and/or simple amino acids prior to absorption and incorporation into new proteins. In order to confirm this assumption, the Agency requires companies to show that a purified protein preparation of the plant-pesticide is digested in artificial preparations of digestive fluids (i.e., gastric or intestinal fluid). The results of digestion are monitored by gel electrophoresis or by western blot analysis and typically proceed from a single high molecular weight band of a given staining intensity to a less intensely stained high molecular weight band (or no high molecular weight band) with the appearance of lower molecular weight bands. If the

protein is rapidly broken down at full strength enzyme concentrations (i.e., no stained bands at first time sample), these tests are often repeated at reduced digestive enzymes concentrations to more clearly track the actual breakdown products.

Digestibility does not in itself predict whether a protein is going to present a dietary hazard. However, a protein's rapid breakdown does suggest that it does not have unusual persistence in the gut. Digestive stability, especially to low pH and gastric fluids, has been one biochemical characteristic, among several, correlated with some food allergens. On the other hand, it is also known that not all stable proteins in the diet are food allergens. The Agency has registered one plant-pesticide, the Cry9C protein, which appears to be resistant to digestion under artificial conditions. Currently, the Cry9C protein is approved only for animal feed use in corn. The Agency has not decided whether to allow this protein in direct human consumption (food use rather than the current animal feed use only restriction). The Cry9C protein was also the subject of the recent FIFRA SAP on food allergenicity (February 29, 2000). It is important to note that resistance to *in vitro* digestion is not a toxicity endpoint itself, but simply an indication that the protein warrants closer examination and perhaps different types of testing.

#### MODE OF ACTION

A further consideration is the mode of action of the protein against its intended target pest. For example, if the protein is known to bind insect protease enzymes or gut epithelium cells for its activity against the intended target pest, these areas would also be the focus for examining toxicity in the mammalian system. If the introduced protein was shown to have no *in vitro* binding to mammalian proteases or gut epithelium, this would add weight to the suggestion that it would not be toxic to mammals. Other examples include membrane active peptides (i.e., magainins) or ribosome binding proteins which could reasonably be expected to have similar activity in mammalian and target pest systems.

It is well known that many individual proteins have several active binding sites and control regions (i.e., allosteric sites) in their native systems. However, it is not clear in the scientific literature if there are examples of proteins having dramatically different activity in mammalian systems outside of those known in their host/parasite interactions. EPA acknowledges that immunorecognition and allergy are mammalian host responses to proteins that are different than the known mode of action of those proteins when involved in host-parasite interactions in plants. However, the consideration of allergenicity, which was the topic of the February 29, 2000 SAP, should aid in addressing this immunologically-mediated event. EPA is also aware of the controversy surrounding prion-based transmissible, spongiform encephalopathies (e.g., sheep scrapie and mad cow diseases) and the fact that the prion protein (PrP) is an aberrant form of a protein normally found in neural tissue. While the exact function of the normal form of the PrP in neural tissue and the spleen is still a subject of scientific inquiry, no homologous protein is known in non-mammalian vertebrates (except perhaps chickens), lower animals or in plants. More importantly, food derived from plants has not been implicated in the etiology of any prion-based diseases. The AA sequence and often the tertiary structure of the PrP proteins in animals that have been

associated with the prion diseases are known. Given these facts it should be possible to screen introduced proteins by their amino acid sequence to indicate any unlikely prion homologies and potential hazards.

### ACUTE ORAL TOXICITY

The biochemical analyses and the mode of action as discussed above give an indication of potential toxicity. However, the Agency still requires that protein plant-pesticides be tested in an acute oral toxicity study with laboratory rodents to confirm the lack of toxicity suggested by the biochemical analyses. The rationale for this acute test is that EPA does not believe there is a sufficient data base of tests with all possible plant-pesticidal proteins that could be introduced into plants to make a safety decision on biochemical analyses alone. In addition, the maximum hazard dose oral test is believed to address the most significant route of exposure for these protein plant-pesticides. The acute oral test is performed with a single high dose (>2-5g/kg body weight) using purified protein as the test substance. The animals are observed for 14 days to ascertain if any adverse clinical signs occur, then are subjected to gross necropsy. To date the plant-pesticide products EPA has registered have been based on *Bacillus thuringiensis*  $\delta$ -endotoxin proteins. Therefore, all the acute oral toxicity tests for plant-pesticides have been done with these proteins or proteins associated with these. As would be expected from the background information on proteins of known toxicity and the historical data base for microbial products, none of the purified *B. thuringiensis*  $\delta$ -endotoxin proteins tested to date show effects when orally administered at high dose levels. This also confirms what has been observed for the numerous microbial products containing *B. thuringiensis*  $\delta$ -endotoxins. While the registered microbial products based on *B. thuringiensis* have a limited range of expressed  $\delta$ -endotoxins, the infrequent mammalian toxicity (e.g., eye and dermal irritation), that has been seen in these microbial products has not been associated with the expressed  $\delta$ -endotoxins.

A specific feature of the acute oral test is that a very large dose of purified protein must be administered as the test substance. Sufficient quantities of protein cannot reasonably be purified from the plant expressing the protein plant-pesticide. Although one company has provided some toxicity information utilizing a plant-expressed test substance, all companies registering plant-pesticides to date have chosen to produce the test substance in an alternative organism, such as either the source bacterium *B. thuringiensis* or an industrial microbe *Escherichia coli*. In order for the alternative test substance to be useful in a determination of toxicity, the equivalence of the microbially produced test substance and that found in the plant needs to be confirmed.

### TEST SUBSTANCE EQUIVALENCE

The rationale behind the equivalence determination is that, while the genetic code and many features of protein synthesis are similar, some aspects of the protein expression systems differ between plants and bacteria. One of the differences is that post-translational modification more frequently occurs in eukaryotic systems (such as plants) than in bacteria. Post-translational changes, such as the addition of sugar groups, are generally associated with the transport of



proteins outside the cell and may enhance the stability of these excreted proteins to extracellular conditions, such as the presence of proteases and pH fluctuations. It is generally true that glycoproteins are more stable than other non-modified protein forms. It is not clear if excreted glycoproteins are already resistant to environmental stresses due to the pre-existing tertiary structure of the protein or due to the subsequent post-translational modification. More important for the food safety determination is whether or not post-translational differences between bacteria and plants change the toxicity characteristics of the expressed protein.

To date the only protein plant-pesticides that have been examined for post-translational changes have been those from a bacterial source. None of these plant-expressed proteins have been shown to have any definitive post-translational modification, such as added sugar residues. Some registrants have indicated that there are consensus sequences that would indicate that a protein would be glycosylated. Other factors that are examined to establish equivalence are: similarity in amino acid sequence ascertained by N-terminal sequencing for a limited number of residues, identical migration rates in SDS-PAGE analysis, immunorecognition in a Western blot assay and similar bioactivity (i.e., activity against the target pest).

#### ACUTE VERSUS REPEAT DOSE TESTING

One of the features of current biopesticide data requirements that differs significantly from the requirements for conventional pesticides, is the tiering or stepwise progression of the data needed for addressing mammalian hazard identification. Acute and subchronic (90-day) testing is routinely required in the initial tier of testing for conventional pesticides in order to evaluate risk and identify potential target organs to focus on in the required chronic (18-24 months) tests. The situation is somewhat different for biopesticides, which include pesticides using either microorganisms or biochemicals. Subchronic (90-day) testing for biochemical pesticides, which are naturally occurring compounds with a non-toxic mode of action, can be required if residues of the compound are expected to be present in significant amounts in treated food or animal feed. For microbial pesticides, the data requirements are all based on acute, single dose, maximum hazard exposures. However, these single dose exposures are specifically designed to examine pathogenicity and infectivity in addition to toxicity. If toxicity from the microbial pesticide is observed in these acute tests, further testing to classify the toxicity or longer term, repeat dose testing would be triggered.

EPA believes that an approach similar to biopesticides, especially microbial pesticides, is justified for examining protein plant-pesticides. The majority of the exposure should be in the diet<sup>1</sup>. The most protective approach for examining these dietary exposures is to test the purified protein at

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<sup>1</sup>There may be special instances where an inhalation or dermal exposure should be considered such as when a protein related to an aeroallergen is found to be expressed in an anemophilous plant with copious wind-born pollen or significant exposure to grain dust is anticipated.

high doses in an acute test. If the protein has been shown to be structurally (by amino acid sequence homology) or functionally related to a protein known to have longer-term effects in mammalian species, then longer term tests may be justified. Examples of the types of proteins known to have longer-term effects in mammals would be those that inhibit digestive enzymes or that bind essential nutrients so that they are not available. For the most part, proteins that would be expected to be toxic should express toxicity when tested at the high doses required in the acute oral test. The results of repeat dose (30-day) toxicity tests performed to date for both tomato expressing Cry1Ab toxin and corn expressing the Cry9C toxin have confirmed that lack of toxicity in the single high dose acute test indicates that repeated dose studies will also be negative for toxicity (Noteborn et al., 1995; and the data evaluation report available at <http://www.epa.gov/pesticides/biopesticides/cry9c/der-44734303a.htm>). Therefore, if the results of the acute test were negative and no structural relationship was seen with toxins, allergens or other proteins that may have known longer-term toxicity, then no further testing would necessarily be indicated. However, if a protein plant-pesticide were suspected of having a longer-term effect, either by the results of the acute test or by relationship to previously identified, longer-term toxins, the protein would require follow-up in a repeat dose (30 day) testing to verify their safety. EPA believes that the longer term test, if required, should be performed with a diet that properly nourishes the test animal yet contains sufficient amounts of the protein plant-pesticide test substance to provide adequate safety margins. However, EPA believes that safety testing using whole food has severe limitations. One limitation would be the inability to establish a margin of safety for the results given the low level of protein expression in plant tissue. Another limitation would be the lack of appropriateness of the plant tissue as laboratory animal food. It is possible that unforeseen toxicity could result from the plant tissue itself being an unusual component for the test rodent in these studies. The stipulation of using the test substance itself for the repeat dose dietary tests would be a critical factor as production of adequate amounts of pure test substance for these tests bears considerable cost. Any other toxicity tests that may be needed to determine the safety of these products will be developed on a case by case basis.

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## Questions

1. Does the Panel agree that the maximum hazard dose approach is generally adequate to address protein toxicity? If not, what additional and/or other approaches would be appropriate? As an alternative for proteins with low expression in plant tissue, would it be possible to demonstrate an acceptable level of mammalian safety by testing the purified protein at, for example, 10,000 times the expression level in the plant? For proteins that show no toxicity in a maximum hazard dose toxicity study, do the negative results of the maximum hazard dose also address the issue of the potential for toxicity following multiple and/or long-term exposures to these proteins?
2. The Agency believes that longer-term testing is not applicable for digestible protein plant-pesticides that display no toxicity in the acute oral toxicity tests. Under what circumstances, if any, should EPA require repeat dose (30 day) feeding studies for protein plant-pesticides? For repeated dose studies, what animal model test system and anticipated effects or toxic endpoints would the Panel suggest be considered?
3. Assume that repeated dose (30 days) testing is indicated and that the test animal diet is appropriately adjusted to provide a healthy diet. Does the Panel agree that whole-food testing with plant-pesticide containing food products does not provide a means to apply an appropriate margin of safety in these studies? Would the plant-pesticide expressing food crop in question amended with pure protein plant-pesticide to yield a higher dose be an appropriate test substance? Or would the purified protein as test substance alone be more appropriate?
4. What is an accepted method of amino acid homology/similarity analysis that can be used to screen for a protein function like mammalian toxicity? Are there any analyses that examine higher levels of protein organization (i.e., secondary, tertiary and quaternary) that could also be incorporated in these structural comparisons?
5. Are peptide fragments that result from the breakdown of proteins more toxic than the intact proteins from which they originated? Are there examples of post-translationally modified proteins which have different toxicity compared to the non-post-translationally modified proteins?
6. How does the breakdown of proteins differ in infants and individuals with digestive disorders compared to those with "normally" functioning digestive systems? Would risk from breakdown products of an otherwise digestible plant-pesticide protein differ for these digestion impaired individuals compared to that posed by any other digestible proteins in their diet?
7. Other than the predominant oral route of exposure, are there any additional routes of

exposure of concern for the toxicity of plant pesticides? Are there any combinations of different routes of exposure that may result in an enhancement of potential adverse effects? Is so, what tests should be considered to evaluate this combined effect?

8. Does the Panel believe there is any other area of toxicity that should be routinely examined for the safety of protein plant-pesticides? What new areas of enquiry should be considered for research into the safety of protein plant-pesticides?