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Toxicological Considerations for Protein Components of Biological Pesticide Products

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The toxicity of protein components of microbial pesticide products is evaluated at EPA by requiring that pesticide manufacturers conduct a thorough taxonomic evaluation of the active microbial ingredient. The requirement for acute toxicity testing by dosing laboratory animals with the active microbial ingredient and with fermentation growth medium materials provides additional information on the toxicity of protein components of microbial pesticides. The potential for toxicity from proteins associated with contaminating organisms is addressed by use of appropriate quality control procedures to minimize or prevent growth of contaminants and by screening of fermentation batches for known human/mammalian pathogens. These considerations also would apply to any biochemical pesticide that is formed via the growth of a microorganism. If a protein itself is intended for commercial use as an active pesticide ingredient, acute exposure studies and *in vitro* digestibility studies could be done to answer potential concerns regarding toxicity.

INTRODUCTION

Under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), the Office of Pesticide Programs (OPP) at the Environmental Protection Agency has the authority to assure that pesticide use in commerce will not result in unreasonable adverse effects to humans and the environment. Under FIFRA, a pesticide is legally defined as ". . . any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest, or intended for use as a plant regulator, defoliant, or desiccant . . ."

Pesticides can be classified as either "chemical pesticides" or "biological pesticides." Chemical pesticides constitute the majority of currently used pest control agents and include, for example, the carbamate and organophosphate insecticides and the phenoxy and triazine herbicides. Biological pesticides are subdivided into three groups: microbial pesticides, biochemical pesticides, and transgenic plant pesticides. The term "biorational" is no longer used to describe the category of biological pesticides.

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Biochemical pesticides are distinguished from conventional chemical pesticides by their specific nontoxic mode of action to target organisms and their natural occurrence. Chemically synthesized pesticides may fall under the definition of biochemical pesticides if they are structurally similar and functionally identical to a naturally occurring substance. Pheromones, natural plant or insect growth regulators, proteins, and enzymes are examples of biochemical pesticides.

Microbial pesticides contain a bacterium, fungus, virus, protozoa, or alga as the active pesticidal ingredient. The active microbial ingredient may be naturally occurring or may be altered via manipulation of genetic material. Commercial production of microbial pesticides often involves growth in large fermentation vessels to which have been added raw materials containing appropriate growth nutrients.

Transgenic plant pesticides that may come under the regulatory oversight of OPP can be defined as plants genetically altered via introduction of genetic material for the purpose of imparting or increasing the production of a pesticide. The active pesticidal ingredient could be considered the pesticidal substance produced from, or modified by, the introduced genetic material. The pesticide product could include the active ingredient and any other substance directly produced from or modified as the result of the introduced genetic material.

Recently, there has been a renewed interest in the use of biological pesticides as effective pest control agents. The application of modern molecular genetic technologies to move pesticidal genes from one microorganism to a different microorganism, or from a microorganism to a plant, certainly has contributed to this renewed interest. Via recombinant DNA techniques, new microbial pesticides have been developed that can synthesize new pesticidal proteins. There also has been an increased interest in the use of biochemical pesticides, especially insect attractants and repellents. Since 1986, the OPP has reviewed data to support small-scale field testing of approximately 50 transgenic plants with pesticidal activity.

If proteins are to be found at significant levels in pesticide products, it will be as components of biological pesticides. Pesticide products that are derived from the growth of living organisms would obviously contain protein components. Also, all transgenic plant active pesticidal ingredients thus far have been proteins. It is conceivable that a preparation of a purified protein may have potential pesticidal use; however, to date, none of these have been submitted to the Agency for registration.

The objective of this paper is to discuss how protein components of pesticides are evaluated for their potential toxicity to humans and mammals. The discussion will largely center on microbial pesticides since proteins are found in the active pesticidal ingredient and also in formulations of the active ingredient. A complete description of all data requirements and study protocols for microbial pesticides, and when these data are required, is presented in the EPA's "Subdivision M of the Pesticide Testing Guidelines" (July 1989).[2]

Considerations given to the potential for toxicity from proteins in microbial pesticides also would apply to biochemical pesticides that are derived from the growth of a living organism. Approaches that the Health Effects Division in the OPP may use to evaluate the toxicity of transgenic plant pesticidal proteins are published elsewhere (McClintock *et al.*, 1991).

[2] Copies available by requesting Document No. PB89-211676 from the National Technical Information Service (NTIS), 5285 Port Royal Rd., Springfield, VA 22161. Telephone No. (703) 487-4650.

PROTEIN COMPONENTS OF MICROBIAL PESTICIDES

Proteins in microbial pesticides can be found as components of the active microbial ingredient or of contaminating microorganisms, or in the growth (fermentation) medium either as added nutrients or as released by metabolic activities or via lysis of the microorganisms (Table 1). Specific proteins associated with the active microbial ingredient may be the pesticidal material. The best example of these are the proteins from varieties of *Bacillus thuringiensis* which are toxic to lepidopteran, dipteran, or coleopteran insects. Other pesticidal microorganisms are active against their hosts because they possess a complement of factors which allow for the development of a parasitic relationship. These factors may include motility and attachment organelles, toxins, capability for utilization of specific compounds as nutrients, detoxification activities, and antibiotic resistance factors. Many of these factors are proteins or result from the activity of enzymes.

Microorganisms also contain proteins which are not required for any host:parasite interaction, but rather for maintenance of cell structure (peptides in bacterial cell walls, virus coat proteins, mitotic apparati in eucaryotes, flagella, and cilia). In addition, microorganisms, like all cells, require a complement of enzymes that allow for catabolic and anabolic functions. The presence of these proteins in microbial pesticide products not only may be from the active microbial ingredient but also could be found if pesticide products become contaminated with other microorganisms.

Microorganisms release certain enzymes that perform activities in the extracellular environment. Intracellular soluble proteins also are released to the growth medium upon cell lysis. An additional source of proteins in growth media is provided when plant or animal raw materials are used as nutrients for growth of a microbial pesticide. These proteins also may be altered during growth of the active microbial ingredient.

Certain microorganisms are not easily grown apart from host cells or host organisms. Viruses are obligate intracellular parasites and often are propagated *in vitro* in cell

TABLE 1

PROTEIN COMPONENTS OF MICROBIAL PESTICIDES

I. Proteins/peptides associated with active microbial ingredient
   A. Nonpesticidal proteins
      1. Intracellular enzymes/proteins for growth and maintenance
      2. Structural cellular components
      3. Toxins, virulence factors, attachment organelles
   B. Pesticidal proteins
      1. Specific protein identified as active pesticidal agent
      2. Other proteins involved in control of target pest
II. Proteins in growth (or fermentation) medium
   A. Extracellular enzymes from active microbial ingredient
   B. Proteins released upon lysis of active microbial ingredient
   C. Proteins from contaminating organisms
   D. Plant, animal, or insect proteins which comprise components of growth medium
      1. Deliberately added as nutrients prior to growth of active microbial ingredient
      2. Modified/altered during growth of active microbial ingredient
      3. Protein components of plant, animal, or insect host when these organisms are required for growth of a microorganism that is an obligate parasite

culture or, where cell lines are not yet available, are reared *in vivo* in the living host organism. Examples of the last are granulosis viruses of certain lepidopteran insects and the entomopox virus of grasshoppers.

TOXICOLOGICAL EVALUATION OF PROTEINS IN MICROBIAL PESTICIDES

The information/data required by the EPA for experimental use or for registration of microbial pesticides do not involve the specific isolation of protein components for evaluation of toxicity effects. Instead, the potential for toxicity of protein components is determined by testing of the active microbial ingredient together with the fermentation medium in laboratory animals. In addition, the Agency also requires that manufacturers of microbial pesticides submit a thorough taxonomic characterization of the active microbial ingredient, as well as a description of the manufacturing (growth) process, including those measures taken to minimize the presence of contaminating organisms. Newly prepared batches or lots of manufactured microbial pesticides are required to be screened for the presence of certain types of human pathogens, and, with *B. thuringiensis* products, by subcutaneous injection of newly prepared batches into rodents (as described in 40 CFR 180.1011).

It is relevant here to discuss some of the types of important information in Subdivision M guidelines, with the objective of evaluating potential toxicity from exposure to protein components of microbial pesticides.

Taxonomy

A pesticide manufacturer must provide to the Agency a thorough taxonomic characterization of the active microbial ingredient using currently acceptable methods. This information allows for a conclusion on whether the microorganism is a recognized human pathogen.

It is doubtful whether pesticide manufacturers would pursue registration of a known human pathogen, although theoretically, certain mammalian pathogens might be useful as pesticides. Also, it might be envisioned that recombinant DNA technologies could be used to "disarm" a known human pathogen of its virulence factors. Nevertheless, a large majority of microorganisms being developed as pesticides—upon taxonomic characterization–either will be recognized as nonpathogenic to humans or will be microorganisms for which microbiologists have little historical experience on potential effects from significant human exposure.

In addition, there are certain microorganisms that are not readily amenable to adequate characterization from standard taxonomic procedures, because: (1) they cannot be grown in pure culture (i.e., contaminating microorganisms are impossible to remove); (2) they can only be grown in association with a particular host organism; or (3) the system of taxonomy used is based on morphological characteristics and the microorganism under consideration has few to no unique morphological structures.

Therefore, because historical experience often is lacking on adverse effects that might occur when humans are exposed to high numbers of environmentally isolated microorganisms, the Agency requires a battery of acute pathogenicity/toxicity studies in laboratory animals.

Toxicity Testing of Microbial Pesticides in Laboratory Animals

In the acute pathogenicity/toxicity studies, the mouse or the rat is exposed to a single high dose level of the microorganism. Three studies are required, reflecting three routes of exposure: oral, pulmonary, and intravenous (or intraperitoneal for larger microorganisms). After dosing, test animals are evaluated by determining mortality, determining body weight gain, making cage-side observations for clinical signs of toxicity, performing a gross necropsy, and evaluating the pattern of clearance of the microorganism from the animals. For the last endpoint, the microorganism is periodically enumerated from appropriate organs, tissues, and body fluids of test animals.

The information from these acute toxicity studies allows an assessment for the potential of the microorganism to be pathogenic, or toxic, to mammals. In most cases, a lack of adverse effects allows for the reasonable conclusion that the protein components of the microorganism are not toxic to mammals.

If toxicity is observed in the test animals—in the absence of signs of pathogenicity—then the toxic components in the test material are to be identified and, to the extent practical, isolated. Further testing in laboratory animals with the toxic components usually will be required to provide an estimation of the amount of material needed to elicit toxic or lethal effects.

The potential toxicity of proteins in the growth or fermentation medium can be evaluated by including the growth/fermentation materials in the dosing material for the acute oral, pulmonary, or intraperitoneal studies. Therefore, it is appropriate to dose animals with fermentation material, which also contains the active microbial ingredient, after growth of the microorganism. It is important, however, to enumerate the number of microbial units (e.g., colony-forming units) in the dosing material. It is often inappropriate to include significant amounts of fermentation ingredients when dosing rodents via the intravenous route, since lethality from nonspecific toxicity may occur. For example, particulates in the fermentation material may result in mechanical blockage of capillaries. On some occasions nonspecific toxicity may result from reaction to injection of significant amounts of foreign protein into the bloodstream. Also, it should be expected that intravenous injection of large numbers of gram-negative bacteria would cause acute mortality from shock reaction to the lipopolysaccharide (endotoxin) component of cell wall material.

Hypersensitivity (i.e., dermal sensitization) studies are not required for registration of microbial pesticide products. It can be expected that injection induction and challenge with foreign proteinaceous components of microbial pesticides into the commonly used laboratory animal (i.e., guinea pig) would yield a positive response. On the other hand, topical induction and challenge with microbial pesticides would most likely lead to a negative response. This, coupled with the historical experience with fermentation products, has allowed for the conclusion that reporting of observed allergic responses to microbial pesticides during manufacture and use should be adequate to address the potential for risk.

Description of Manufacturing Process

While the data on the taxonomy and on the acute toxicity studies provide information useful in assessing the toxicity of protein components of the active microbial

ingredient, it is information on the manufacturing process that addresses the likelihood of toxicity that might occur from the presence of contaminating organisms. Particular attention is given to the measures that pesticide manufacturers use to minimize the potential for growth of contaminating organisms. A description of the quality control procedures used should include analyses of stock cultures and "seed" cultures for biological purity, description of sterilization procedures for growth media and for fermentation vessels, monitoring of appropriate physical conditions during fermentation, and analysis of lots when fermentation is completed. The Agency requests that the pesticide manufacturer present this information as it provides a framework for a discussion on the likelihood of the presence of toxic or sensitizing materials arising from growth of contaminating microorganisms in the pesticide product.

Each newly produced batch of microbial pesticide can be analyzed for key human pathogen classes and for unexpected toxins via injection into laboratory animals. For *B. thuringiensis* fermentation batches, each lot is to be tested ". . . by subcutaneous injection of at least 1 million spores into each of five laboratory test mice." The test results should show ". . . no evidence of infection or injury in the test animals when observed for 7 days following injection" [40 CFR 180.1011]. The Agency currently is reevaluating whether or not the subcutaneous injection test should be replaced with an intraperitoneal injection test. For reregistration of active *B. thuringiensis* ingredients, an intraperitoneal injection screen is required in which mice (five male and five female mice/dose level) are injected with 10^6, 10^7, and 10^8 units of *B. thuringiensis*. As with the subcutaneous test, animals are observed for toxicity and mortality for 1 week.

No microbial pesticide products are to contain human pathogens (such as *Shigella, Salmonella,* and *Vibrio*) at hazardous levels. If the production method can support growth of human or animal pathogens then each production batch should be tested for their presence. At present, applications for registration of microbial pesticides should contain an analysis of significant mammalian pathogens that might be present. The applications also should state proposed methodologies for detecting these pathogens and/or eliminating them from the product if the product is not to be discarded.

TOXICOLOGICAL CONSIDERATIONS FOR PROTEINS THAT ARE ACTIVE PESTICIDAL INGREDIENTS

It is conceivable that a pesticide manufacturer may wish to register a pesticidal active ingredient that is itself a protein and in a situation where the protein is not associated with a microorganism, plant, or animal. Although no such product has yet been submitted for registration, some general scientific considerations on evaluating the toxicity of protein pesticides can be offered. Uncharacterized proteins from plant, animal, and microbial sources are significant dietary sources of carbon, nitrogen, and energy. Proteins are susceptible to acid and enzymatic digestion to amino acids prior to assimilation. Certain proteins are known to be toxic to humans, and these have received wide study. Available information should be useful for predicting the potential for toxicity of many proteins. If toxicity testing of a protein is considered necessary, then acute exposure studies in laboratory animals should be sufficient, since—if toxic—proteins are known to act via acute mechanisms. Also, laboratory animals show acute toxic effects from exposure to proteins known to be toxic to humans. Modern molecular methodologies can be applied to design novel proteins, including some that might be

more efficacious as pesticides than their naturally occurring counterparts. It might be appropriate, also, to use *in vitro* digestibility studies with novel proteins to determine whether the structural changes made might significantly affect the rate of degradation in the digestive tract.

SUMMARY

At the U.S. EPA, pesticides designated as "biological pesticides" include microbial pesticides, biochemical pesticides, and transgenic plant pesticides. The active ingredient in a microbial pesticide may be a bacterium, fungus, virus, protozoan, or alga. Biochemical pesticides are naturally occurring pesticides that control a target pest by a nontoxic mode of action and include products such as pheromones and natural plant and insect growth regulators. Transgenic plant pesticide products could include pesticidal and other materials produced from, or modified directly by, genetic material that is introduced into a plant.

If proteins are found at significant levels in pesticide products, it would be as components of biological pesticides. Protein components of microbial pesticide products can be used to illustrate how the Health Effects Division of the Office of Pesticide Programs, U.S. EPA, addresses potential toxicity effects of proteins on humans and mammals.

Pesticide manufacturers are required to submit thorough taxonomic information on the active microbial ingredient. Also, submitted are acute oral, pulmonary, and intravenous (or intraperitoneal) toxicity studies with the active microbial ingredient tested in laboratory rats or mice. Scientific review of these data allows for conclusions on the toxicity of proteins associated with the microbial component of the pesticide product. Proteins in the microbial growth (i.e., fermentation) medium are evaluated for toxicity by including the fermentation medium as a component of the dosing material in the acute toxicity studies.

Pesticide manufacturers also are required to use appropriate quality control procedures during the production/growth of microbial pesticides so as to minimize the potential for growth of contaminating microorganisms. Newly grown batches of microbial pesticides can also be evaluated for the presence of acutely toxic protein components or for pathogens by injection into test mice. Conventional microbiological methods also can be employed to screen fermentation batches for classes of significant human pathogens.

If modern molecular methodologies are applied to design novel pesticidal proteins, then the additional application of *in vitro* digestibility studies may be useful to predict if the structural changes made might cause concern regarding toxicity.

REFERENCES

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