Basic Techniques in Studying Mycotoxins: Isolation of Mycotoxins

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ABSTRACT

The basic techniques used and the potential problems encountered in isolation of mycotoxins from naturally contaminated commodities and fungal cultures are discussed. Specifically presented are the factors to be considered in selecting a bioassay organism, methods of preparing formulations for mycotoxin administration, modes of administration, methods available for purification, and problems encountered during purification.

Acute clinical syndromes and deaths caused by mycotoxins may be diagnosed or suspected by veterinary diagnosticians. However, it is believed that in many instances the chronic or nonlethal effects of mycotoxins are not recognized. These latter effects, although frustrating to the veterinary diagnostician, may be economically devastating to the producer through losses incurred from lower productivity, reduced weight gains, and impaired resistance to infection (5-10).

The techniques employed to detect and isolate mycotoxins depend largely on the chemical and physical nature of the toxin involved, the nature and number of contaminants, and the titre of the toxin being produced by the particular fungus.

Two general approaches to detection and isolation of mycotoxins have been used. The first is the consequence of a natural outbreak of suspected mycotoxicosis similar to that which occurred in England with the aflatoxins (4). The clinical signs in this case were acute and resulted from a heavily contaminated feed source. In the other approach to detection and isolation of mycotoxins, the fungi that commonly invade an agricultural commodity are evaluated for toxin-producing potential (2). This latter approach is indirect and it attempts to identify or predict mycotoxin problems that might be occurring on a chronic level. Once the potential for toxin production and the incidence of particular toxin producers have been determined for a commodity, analytical techniques can be developed to determine whether or not those toxins occur naturally in that commodity.

ISOLATION OF MYCOTOXINS FROM COMMODITIES SUSPECTED OF CAUSING NATURAL MYCOTOXICOSES

Before initiating in-depth analyses for mycotoxins in a suspect commodity, it is best to ascertain that mycotoxins are the probable cause of illness. This can be determined by elimination of other causes at necropsy, and/or on-site inspection. Mycotoxicoses are best confirmed when identical clinical signs are produced when the commodity is fed to an experimental animal, preferably of the same species involved in the initial outbreak. Since one of the first inclinations is to destroy suspect feed, a problem frequently encountered is the non-availability of adequate amounts of the suspect feed for feeding trials and analyses.

It is best, if sufficient contaminated material is available, to isolate and identify the toxin causing the problem before determining which fungus is responsible for toxin production. It is not difficult to isolate several toxigenic fungi invading a particular lot of commodity; therefore, to prevent confusion, it is better to first isolate the toxin involved, then determine which fungus has produced the toxin. Again, this was true with the aflatoxins which were isolated from contaminated groundnut before determining which fungus produced the toxins (4).

ISOLATION OF MYCOTOXINS FROM CULTURES OF FUNGI CONTAMINATING A PARTICULAR COMMODITY

This approach involves screening a commodity for toxigenic fungal isolates, followed by isolation and identification of the toxic metabolites (1). The relative frequency of invasion by individual toxin-producers is determined in samples of the commodity from various sources. If the commodity proves to be an adequate substrate for toxin production by the fungi found most often invading the commodity, analytical techniques can
then be developed to determine if the toxin occurs as a natural contaminant of the commodity.

**SELECTION OF BIOASSAY ORGANISMS**

Several factors must be considered in selecting a bioassay organism for isolation of mycotoxins. These include availability, economics, and validity of the bioassay. An effective and economical small animal bioassay organism can be obtained from chick hatcheries producing the Leghorn breed which are used exclusively for egg laying. The Leghorn cockerels are of no economic value and, therefore, can be purchased at a fraction of the cost of other bioassay animals. These are readily available in large numbers at frequent intervals and provide a sensitive bioassay if used before 2 days of age, preferably at 1-day-old and before being put on feed. Other small animals such as mice, rats, hamsters, etc. may be used if they are available; however, these are more expensive than day-old-cockerels. Use of non-vertebrate bioassays (bacteria, brine shrimp, etc.) are not valid in isolation of vertebrate toxins since there is little correlation between vertebrate toxicity and toxicity in microorganisms and invertebrate animals. In addition, results from administration of metabolites via routes other than oral are often confusing since a metabolite must be toxic orally to a vertebrate animal to be considered a mycotoxin. Use of chick embryo and other injection-type bioassays for the initial isolation of mycotoxins are, therefore, not recommended.

**FORMULATIONS FOR ADMINISTRATION OF MYCOTOXINS**

The suspect commodity or the fungal culture being tested may be dried, ground, and fed ad libitum to test animals. However, the grinding, mixing and feeding of dried fungal-invaded substrate may present a problem concerning worker safety. Without elaborate precautions, airborne spores and microparticles are unavoidable when samples of dried, melted substrate are prepared. Since fungal spores may contain toxins and be allergenic, dangers from exposure to this material must be a primary consideration. Also, these fungi are mainly saprophytic but some species (e.g. *Aspergillus fumigatus*) can be pathogenic to man (3).

Health hazards can be alleviated by autoclaving and/or extracting the fungal cultures or moldy substrate with an appropriate solvent such as chloroform. Addition of solvents such as chloroform to the culture flasks or moldy substrate and subsequent heating to reflux results in complete wetting and precipitation of airborne spores within the container and destruction of the viability of the spores. The investigator can then complete the remaining steps without risk from airborne fungal spores and microparticles. This procedure should be given serious consideration because extraction is the first step in isolation of the toxin.

Inert carriers that can be used in preparing mycotoxin formulations are vegetable oil, Tween 20, and propylene glycol. Although most mycotoxins are poorly soluble in these carriers, a suitable preparation can be made by first dissolving the mycotoxin in an appropriate solvent, adding the solution to the carrier and removing the solvent under vacuum (2).

At dosage levels up to 15 mg/ml, the mycotoxin generally will remain in solution and provide an excellent surface area for absorption in the experimental animal. In some instances, combinations of the above carriers will give better solubility than any used alone.

The relative toxicities of these carriers to day-old-cockerels dosed orally via crop intubation at 1 ml/chick in order of increasing toxicity are vegetable oil, Tween 20, and propylene glycol. One-day-old cockerels show no adverse effects when dosed orally with 1 ml of vegetable oil, minor effects with 1 ml of Tween, and 1 ml of propylene glycol is acutely toxic to day-old cockerels.

**PURIFICATION OF MYCOTOXINS**

Techniques used in purification of a particular mycotoxin monitored by bioassay depend largely on the nature of the mycotoxin involved. An example of a technique that can be used is as follows: The toxin is extracted with an appropriate solvent and fractionated with any number of column adsorbents (e.g. silica gel, florisil, etc.). The typical elution series for a crude chloroform extract on a silica gel column could be as follows: hexane, benzene, ethyl ether, chloroform, ethyl acetate, acetone, and methanol (three column volumes of each eluent). A second column with a linear gradient elution series and a fraction collector could then be used. The linear gradient elution selected depends on which gross fraction contains the toxin. If the toxin was in the ethyl acetate fraction, a linear gradient from chloroform to ethyl acetate, benzene to ethyl acetate, or from ethyl ether to ethyl acetate may be appropriate. If purification is inadequate, additional adsorbents such as florisil, alumina, ion-exchange resin, or molecular sieve chromatography may be used. Preparative thin-layer chromatography and high pressure liquid chromatography are excellent techniques, if available. Mycotoxins that are polar in nature (i.e. soluble in methanol and/or water) are not amenable to all types of adsorbents. Aqueous adsorbents available are molecular sieve, ion-exchange and DEAE cellulose for conventional column chromatography.

**POTENTIAL PROBLEMS ENCOUNTERED DURING TOXIN PURIFICATION**

1. Decreased solubility of the toxin as a function of purity and, therefore, decreased absorption and loss of toxicity. The metabolite may be toxic in crude preparations but relatively non-toxic in pure form due to decreased solubility. A recent example of this in our laboratory was encountered during the isolation of chaetoglobosin C (Unpublished) and, to some extent, with flavutoxin (1).
2. Instability of the toxin as function of purity. The toxin may be quite stable in crude preparations but highly unstable in pure form.

3. Loss of toxicity during purification due to removal of synergistic metabolites and/or due to removal of companion toxins acting in concert.

4. Loss of toxin-producing potential by the mold as a result of subculture or "domestication."

Those problems could occur individually or simultaneously to various degrees.

Isolation of each mycotoxin is a unique challenge. The isolation may require crystallization from crude extracts or the use of several combinations of sophisticated techniques which may be frustrated by failure due to the presence of stubborn impurities or to instability of the toxin in pure form.

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Mention of firm names or trade products does not imply endorsement or recommendation by the U.S. Department of Agriculture over other firms or similar products not mentioned.

REFERENCES