Importance of Aflatoxins

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ABSTRACT

Aflatoxins are toxic secondary metablites produced by some certain strains of *Aspergillus flavus, A. parasiticus, A. nomius, A. tamarii* and a new aflatoxigenic species of *Aspergillus, A. bombycis.* These toxins are both acutely and chronically toxic in animals, including man, producing four distinct effects: acute liver damage; liver cirrhosis; tumour induction and teratogenesis. Aflatoxins have been recognized as worldwide health hazard to humans and domesticated animals and they are among the most potent mutagenic and carcinogenic compounds known to be produced in nature. This article reviewed the importance of aflatoxins and their controls.

1. INTRODUCTION

Aflatoxin was first discovered in 1960 when there were mass deaths from liver disease of turkeys in Norfolk, UK followed by deaths of other farm animals [1]. More than 100,000 turkeys died within a few months. Scientist called the new disease "Turkey X Disease" because they did not know its cause. It was finally established that all birds affected had been fed with feed prepared with contaminated groundnut meal [2]. Examination of the incriminated groundnut meal revealed the presence of mould mycelium and thin layer chromatography showed the presence of several new fluorescent compounds [3]. The mould was shown to be *Aspergillus flavus* and the toxin was named aflatoxin.

Since this discovery, the aflatoxins have been recognized as a worldwide health hazard to humans and domesticated animals. They are among the most potent mutagenic and carcinogenic compounds known to be produced in nature.

The aflatoxins are a group of toxins having similar molecular structures. Although 17 compounds, all designated aflatoxins, have been isolated, the term aflatoxins usually refers to four compounds of the group of bis-furano-coumarin metabolites named B1, B2, G1 and G2 [4]. The four substances are distinguished on the basis of their fluorescent colour, B standing for blue and G for green. Aflatoxins found most abundantly in foods derived directly from plants are the B and G aflatoxins. Aflatoxin B1 is produced most abundantly, and is also the most toxic, followed by G1 while B2 and G2 occur in lower concentrations. The aflatoxins are intensely fluorescent, when exposed to long-wave ultraviolet light so that it is possible to detect these compounds at extremely low levels (ca. 0.5 ng or less per spot on thin layer chromatograms).

Aflatoxins are freely soluble in moderately polar solvents such as chloroform and methanol and especially in dimethylsulfoxide. The solubility of aflatoxins in water ranges from 10-20 mg/l. As pure substances, the aflatoxins are very stable at high temperature, when heated in the air. Little or no destruction of aflatoxins occurs under ordinary cooking conditions, and heating for pasteurization. However, roasting groundnuts appreciably reduces the levels of aflatoxins and they can be totally destroyed by drastic treatment such as autoclaving in the presence of ammonia or by treatment with hypochlorite [4].

After consumption by an animal, the B aflatoxins are metabolized to the M aflatoxins (aflatoxins M1 and M2 are hydroxylated metabolites of aflatoxins B1 and B2). Aflatoxin M1 is of special interest because it can be transmitted to newborn offspring in the mother's milk [2]. Aflatoxin M1 is less toxic than aflatoxin B1, but nevertheless poses a particular threat to the newborns because they are more susceptible to the effects of aflatoxins than are older individuals. Humans are not only at risk from eating contaminated foods derived directly from plants, but also from drinking milk from animals that had consumed such foods because aflatoxins can be transmitted in milk. Aflatoxin M1 remains stable even after milk is processed into dried milk, cheese or yoghurt [2]. Aflatoxin M1 has been reported in human's milk. 99.5 % of breast milk from 445 people in Abudabi were found to contain 2-3 ng/l of aflatoxin M1 [5]. However, 43 samples of human breast milk collected from 3 hospitals in Bangkok were not contaminated with aflatoxin M1 [6].

Production of aflatoxin

Aflatoxin contamination of foods and feeds occurs when aflatoxigenic species of the *A. flavus* group successfully colonize and grow in a commodity, and subsequently produce the aflatoxin secondary metabolites. The species of the *A. flavus* group that produce aflatoxins include *Aspergillus flavus*, *A. parasiticus, A. nomius*, *A. tamarii* and *A. bombycis* [7, 8, 9]. *Aspergillus flavus* produces the B aflatoxins (B1 and B2) while *A. parasiticus* produces aflatoxins B1, B2, G1 and G2. *Aspergillus nomius* which was first isolated from bee in USA also produces aflatoxins B1, B2, G1 and G2 [10]; *A. nomius* has also been isolated from corn in the north of Thailand. Some strains of *A. flavus* have been re-identified as *A. parasiticus* and *A. nomius*. *Aspergillus tamarii,* which was first isolated from soil collected from a tea (*Camellia sinensis*) field in Japan, produces aflatoxins B1 and B2.

The toxigenic *A. toxicarius* Murakami was considered synonymous with *A. parasiticus*. The other species in the *A. flavus* group, including the domesticated Koji moulds *A. oryzae* and *A. sojae,* do not produce aflatoxins [11]. *Aspergillus oryzae* and *A. sojae* are genetically identical with *A. flavus* and *A. parasiticus*. Although the recent molecular data are recognized, discussions at the Third International Conference on *Penicillium* and *Aspergillus* have resulted in the acceptance of the names *A. sojae* and *A. oryzae* [12].

Aflatoxins can be produced only under particular environmental conditions. Therefore, the actual growth of aflatoxigenic fungi on the food does not necessarily mean that aflatoxins are also present. Moisture, temperature, and insect or other injury as well as the *A. flavus* isolate, the crop and the environmental conditions are particularly important factors in determining whether aflatoxins are actually produced as the fungus grows within the seeds or grains [2,7]. In temperate regions *A. flavus* is likely to be responsible for aflatoxin production whereas *A. parasiticus* is more active in subtropical and tropical regions. Both species of *Aspergillus* commonly occur as soil saprotrophs, or among the fungi present on grains and other foods during storage. These fungi can live parasitically on growing plants under particular conditions. *A. flavus* is known to be able to infect the seeds of peanuts, cottonseed, and corn kernels as they are developing in the field. Invasion of the seed or grain can occur while the plant is maturing in the field. Aflatoxins can be produced in preharvest as well as in stored products [7]. Spores of *A. flavus* can be introduced into the plant through insect wounds, or they can germinate on the pistil of the flower; the spores also contain aflatoxin [13]. After the spores have germinated, the mycelium invades the developing embryo. There, the fungus grows essentially as a pure culture, without competition from other fungi. Mycelial growth and aflatoxin production can continue after harvest so long as moisture levels remain high within the seeds or grains. Aflatoxin production is particularly favoured by very moist conditions. As the grains are dried further for storage, additional aflatoxin production is unlikely. Aflatoxins are produced only between temperatures of 12 and 42 °C, although the fungus itself can grow at the higher temperatures that often develop as a result of respiration occurring during storage. Aflatoxin production is further encouraged in the absence of competing fungi [7].

Aflatoxin analysis

All analytical methods for aflatoxins include 3 steps, i.e, sampling and sample extraction, clean-up and purification and determination of toxin.

Chemical methods involve the extraction of samples with polar solvents, partial purification by passage through a silica gel column or proprietary separating pack, concentration and detection by thin layer chromatography (TLC) or liquid chromatography (LC) [3].

Immunochemical methods are of 3 types, i.e., radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and immunoaffinity column assay (ICA). The first two methods are based on competition between the unlabeled aflatoxin in the test solution and the labeled aflatoxin in the assay system for the specific binding site of antibody molecules. Radioactive aflatoxin is used as a labeled ligand in the RIA and an aflatoxin-enzyme conjugate is used as ligand in the ELISA. In the ICA procedure, the antibody column traps or binds the aflatoxins, which then can be eluted from the column with methanol for subsequent measurement [14].

Although commercial ELISA kits are suitable for aflatoxin monitoring and for testing agricultural exports or imports, their high cost may restrict their use in most analytical laboratories in developing countries [15].

Control of aflatoxin producing fungi and aflatoxin production

Every year a significant percentage of the world's grain and oilseed crops is contaminated with hazadous mycotoxins such as aflatoxin [16]. The presence of aflatoxins in animal feeds results in substantial economic losses to poultry and livestock industries. The prevalence of aflatoxins in a variety of foods destined for human consumption is also a major concern. Aflatoxins are heat stable and survive a variety of processing procedures. These compounds have been found as natural contaminants in food crops and foods such as peanut butter and other peanut products, corn and cornmeal, breakfast cereals, dairy products and other processed foods. Aflatoxin B1 has been implicated as a factor in human liver cancer and classified as a probable human carcinogen. Physical, chemical and biological methods have been investigated in order to prevent the growth of aflatoxigenic fungi, eliminate or reduce the toxin levels, degrade or detoxify the toxins in foods and feeds. Some chemicals, herbs and microorganisms have been studied for the control of aflatoxin producing fungi and aflatoxin production in agricultural commodities. Details are as follows:

1) Chemical methods

Chitosan

Chitin, poly- β-(1,4)-N-acetyl-D-glucosamine, a cellulose-like biopolymer, is distributed widely in nature such as in marine invertebrates, insects, fungi and yeasts [17,18]. Chitosan, a deacetylated derivative of chitin, is less dominant but is found in the cell walls of some fungi [17, 19]. Chitosan has antifungal properties [20] and it has been used in medicine, cosmetics, biotechnology, water treatment, agriculture and food production. Chitosan was found to inhibit the growth of and aflatoxin production by *A. parasiticus* when used at a concentration of 3 mg/ml on preserved kumguat [21]. Chitosan at 0.1-0.5% did not inhibit the growth of *A. parasiticus* in rice when incubated for 7-14 days. However, the production of aflatoxin was reduced when 0.2% of chitosan or more were applied whereas chitosan at 0.1% stimulated the production of aflatoxin B and G from 441.35 to 812.22 μg/g. It was noted that no aflatoxin B1 was produced when 0.3-0.5% of chitosan were added to rice [22]. Chitosan seemed to be more effective at inhibiting toxin production than inhibiting fungal growth [21].

Salts

Sodium chloride (NaCl), ammonium carbonate ($(NH_4)_2CO_3$) and sodium bisulphite ($NaHSO_3$) have been used to control the growth of *A. parasiticus* and aflatoxin production in irradiated corn. Sodium chloride at 4 and 5%, ammonium carbonate and sodium bisulphite at 2% or more could inhibit the growth of the fungus and aflatoxin production completely when the cultures were incubated at room temperature for up to 28 days. Low concentrations of sodium chloride stimulated aflatoxin production whereas high concentrations inhibited fungal growth and aflatoxin production [23, 24]. It has been postulated that high concentrations of sodium chloride adversely affect the water activity required for growth and toxin production [25] or that sodium ions interfere with ion transport in the organism [26]. The inhibition of fungal growth and hence, the toxin production by ammonium carbonate might be due to the conversion of carbonate ions into free carboxyl groups which kill the fungus [27]. Sodium bisulphite may inhibit fungal growth by the reaction of sulfuric acid with fungal enzyme. Sodium bisulphite also reacts with aflatoxins under various conditions such as temperature, concentration, and time to form water-soluble products.

Herbs

Powders and extracts of various herbs, spices and essential oils have been reported to have antimicrobial activity and some of them also inhibit aflatoxin formation [28, 29, 30]. Crude extracts of lime, red onion, garlic, ginger, clove, carrot and neem tree at various concentrations have been tested for inhibitory effect on aflatoxin producing fungi such as *A. flavus* and *A. parasiticus*. Garlic was found to be the best herb for inhibiting the growth of aflatoxin producing fungi.

Essential oils from some Thai herbs at various concentrations are under investigation in the author's laboratory for their inhibitory effect on growth and aflatoxin production of *A. flavus* and *A. parasiticus* in Potato Dextrose Agar and corn (unpublished data). Preliminary results indicated that at certain levels of essential oils applied to the cultures, the growth of fungi was inhibited for a certain period. Further studies are planned.

2) Biological methods

Many microorganisms including bacteria, actinomycetes, yeasts, moulds and algae show varying abilities to degrade aflatoxin [3]. *Flavobacterium aurantiacum* (NRRL B-184) was shown to remove aflatoxin from a liquid medium significantly without the production of toxic by-products [31] and has also been reported to remove aflatoxin B1 from peanut milk [32]. Some microorganisms are reported to be able to compete and inhibit the growth of aflatoxigenic fungi. Nontoxigenic strains of *A. parasiticus* and *A. flavus* fungi were used to compete with toxin producing fungi in preharvest peanuts and cottonseeds. Initial studies showed that bioprevention can be used to reduce preharvest aflatoxin contamination in peanuts and cottonseeds significantly [33].

Bacillus subtilis, a bacterium isolated from groundnuts, was found to inhibit the growth of *A. flavus* in groundnuts [34]. Sommartya *et al.* [35, 36] showed that mixing *B. subtilis* with groundnuts could reduce the damage caused by *A. flavus*.

Streptococcus lactis (cheese starter) was found to inhibit aflatoxin production by *A. parasiticus* [37]. The experiment was performed in order to study the interaction between *S. lactis* and *A. parasiticus* on production of aflatoxin in liquid medium and to compare the ability of *S. lactis* with other lactic acid bacteria used in commercial yoghurt to detoxify aflatoxin B1. The inoculation of *S. lactis* in a mixed culture with *A. parasiticus* and into a 3-day-old *A. parasiticus* culture reduced aflatoxin accumulation from 108.33 to 94.18 and 31.01 μg/ml, respectively, after two days of incubation [38]. When *S. lactis* was cultured for 3 days before the inoculation of *A. parasiticus* spores, it was found that *A. parasiticus* could produce only 58.01 μg/ml of aflatoxin on day 2. *Streptococcus lactis* was further examined for the ability to detoxify aflatoxin B1 in yoghurt in comparison with lactic acid bacteria from commercial yoghurt. *Streptococcus lactis* could detoxify aflatoxin B1 from 50 to 33.70 μg/ml whereas bacteria from commercial yoghurt detoxified aflatoxin B1 from 50 to 37.25 μg/ml after 7 days of storage in the refrigerator.

Various investigators have reported that a number of microorganisms affected the production of aflatoxin in a competitive environment. A mixture of *Lactobacillus* species has been reported to reduce mould growth and inhibit aflatoxin production by *A. flavus* subsp. *parasiticus* [39]. Coallier-Ascah and Idziak [40] found that the inoculation of *A. flavus* into a culture of *S. lactis* in LTB medium resulted in little or no aflatoxin accumulation even though the growth of *A. flavus* was not hindered. The aflatoxin production of *A. parasiticus* was less when the mould was grown with *L. casei* [41]. It has been indicated that the presence of another microorganism in the culture can affect the behavior of *A. parasiticus*. Fungal growth and aflatoxin production can be enhanced, retarded or remain unchanged as a result of another microorganism in the environment. The adsorbtion of aflatoxins to the bacterial cell wall has been proposed as the mechanism of aflatoxin degradation by lactic acid bacteria [31, 42]. Lactic acid bacteria in some way might also interfere with the synthesis of aflatoxin [40].

Megalla and Hafez [43] reported that aflatoxin B1 added to yoghurt was completely transformed to a new fluorescing compound corresponding to aflatoxin B2a. The new compound has been referred to as hydroxydihydro-aflatoxin B1. This compound caused no bile duct hyperplasia and no deaths in the duckling test [44].

Rhizopus oligosporus is a fungus used in the preparation of tempeh, a fermented food which is a high nutritional source of protein and vitamins and consumed widely in Indonesia. Tempeh is usually made from various cereal grains but soybean is the most commonly use. Soybean used for the tempeh production may sometimes be contaminated with toxigenic fungi. Thus, the effect of *R. oligosporus* on the growth of *A. parasiticus* and aflatoxin production during tempeh production was investigated. Steamed soybean with the addition of *R. oligosporus* and *A. parasiticus* spores did not contain aflatoxin whereas steamed soybean inoculated with only *A. parasiticus* spores contained aflatoxin [45]. *R. oligosporus* was reported to inhibit the growth and sporulation of *A. flavus* and also aflatoxin [46] ; other species of *Rhizopus*, including *R. oryzae* and *R. chinensis* also showed the same properties. *R. oligosporus* also has other desirable properties that help to ensure safety and tempeh quality [47]. Since *Rhizopus* spp. grow fast and quickly deplete the substrate of fermentable carbon-compounds, they outcompete slower growing fungi such as *Aspergillus* spp. [48]. Furthermore, *R. oligosporus* exhibit antimicrobial activity against some *Bacillus*, *Clostridium*, and *Staphylococcus* spp. [49, 50], and also produce substances that inhibit *A. flavus* growth [48]. A survey of tempeh in Indonesia showed that all products tested were free from aflatoxin [51].

Aspergillus oryzae is the main species that is naturally used in many traditional fermented foods such as soy sauce. Although a number of strains of *A. oryzae* have been shown not to produce aflatoxin [52], it is difficult to distinguish *A. oryzae* from *A. flavus* or *A. parasiticus* for they are genetically identical. Oriental fermented foods can be contaminated with toxigenic fungi. Thus there is a risk of aflatioxin contamination. A study was carried out to determine whether aflatoxin producing *A. flavus*, could grow with *A. oryzae* and produce aflatoxin during the fermentation of soy sauce. Aflatoxin level was decreased from 2200 to 237 ppb when the two fungi were grown together during koji fermentation and a gradual decrease occurred during moromi fermentation (unpublished data). Sardjono *et al.* [53] reported that aflatoxin production by *A. flavus* was reduced during mixed culture with *A. oryzae*. This might be explained by the ability of *A. oryzae* to produce a large amount of organic acids [54].

Ganoderma is a medicinal fungus and has been treasured for this value in China for more than two thousand years [55]. This fungus was described as a nontoxic medicine which was beneficial to viscera and could improve intelligence by enhancing memory, hearing, vision and smell. *Ganoderma lucidum* (Lingzhi mushroom) can be produced in large quantities by solid state fermentation and submerged fermentation. Studies are underway in the author's laboratory investigating the effect of *G. lucidum* mycelial growth on the growth of *A. parasiticus* and its aflatoxin production.

Trichoderma spp. have been investigated as a biological control agent for certain fungal plant diseases. *Trichoderma* spp. are potential candidates for biocontrol of some mycotoxin-producing fungi, but there is some doubt as to their osmotolerance within the air-dry seed [56]. Calistru *et al.* [56] reported that two isolates of *T. harzianum* and two of *T. viride* were capable of inhibiting the

growth of *A. flavus* and *Fusarium moniliforme*. Culture filtrates of *T. viride* and *T. harzianum* were inhibitory to *F. moniliforme* and to a lesser extent, *A. flavus* [57].

2. CONCLUSIONS

Developing comprehensive understanding of both the mycology of aflatoxin contamination in various ecosystems and the molecular regulation of aflatoxin formation is imperative. This understanding ultimately will provide the tools for developing strategies for effective control of aflatoxigenic fungi and elimination of aflatoxin contamination from animal feed and human food chains [58].

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