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Aflatoxin Contamination in Soybeans: Role of Proteinase Inhibitors, Zinc Availability, and Seed Coat Integrity

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Soybean trypsin inhibitors are thought to ward off pathogens. Studies with aflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus*, frequent soybean contaminants, revealed that trypsin inhibitors do not affect the growth of these fungi and aflatoxin production. Further, the availability of zinc, an essential mineral for aflatoxin synthesis that was thought to explain increased aflatoxin accumulation in cooked compared with raw soybeans, was shown to decrease upon cooking. Seed coat integrity, ensuring limited access and a low moisture content, is responsible for the slow colonization of the seed by *A. flavus*.

Soybeans contain considerable quantities of trypsin and chymotrypsin inhibitors (11, 26), namely a high-molecular-weight (Kunitz) trypsin inhibitor (27) and a group of low-molecular-weight inhibitors (Bowman-Birk type), including a double-headed inhibitor of both trypsin and chymotrypsin (18, 34).

In contrast to wound-induced proteinase inhibitors described leguminosae (3), the physiological function(s) of the constitutive soybean proteinase inhibitors is poorly understood. Postulated roles are (i) storage proteins, (ii) regulators of endogenous proteinases, and (iii) protective agents against insects and microbial pathogens (28, 29). The high content of sulfur-containing amino acids suggests that they function as storage proteins, but their insignificant decrease during germination argues against it (11). Rapid release of protease inhibitors from imbibed (19) and germinating (34) seeds as well as a certain suppression of potential contaminants, including an *Aspergillus* species (14), or their proteinases (23, 24) may indicate a role in warding off pathogens.

Soybean trypsin-chymotrypsin inhibitors are antinutritional factors. Although cooking partially eliminates proteinase inhibitor activity and produces acceptable food (20), effort has been directed toward breeding inhibitor-free soybeans (25). If proteinase inhibitors participate in the defence against microbial attack, including attack by toxin-producing species such as *Aspergillus flavus* and *A. parasiticus*, the lack of such inhibitors may increase the risk of seed contamination caused, e.g., during improper storage (high humidity) or through seed damage.

The increased growth of *A. flavus* and *A. parasiticus*, and the correspondingly increased toxin production, after the cooking of soybeans, soybean flour, or soybean-based medium (13, 16, 31) may indicate that heat-labile seed constituents (e.g., proteinase inhibitors) have an antifungal effect. However, this increase has rather been attributed to the availability of zinc, an essential trace element for aflatoxin synthesis (21), and to the presence of phytate (13, 16).

The objectives of the present investigation were to study the potential influence of heat-labile proteinase inhibitors and zinc availability as well as the effect of seed coat integrity on soybean seed colonization by *A. flavus* and *A. parasiticus* and subsequent aflatoxin contamination.

MATERIALS AND METHODS

Soybean trypsin inhibitor (SBTI) (Kunitz type, heat labile) was purchased from Serva, Heidelberg, Federal Republic of Germany (molecular weight, approx. 22,000) and from Boehringer GmbH, Mannheim, Federal Republic of Germany (molecular weight, 20,100); phytic acid from corn (sodium salt) came from Sigma Chemical Co., St. Louis, Mo; a mixture of aflatoxin standards (AFB_1, AFB_2, AFG_1, and AFG_2 in benzene) was obtained from Makor Chemicals Ltd., Jerusalem. Soybeans from the last harvest, cultivar Maple Arrow, were supplied by E. Schweizer, Thun, Switzerland, and were stored at 20°C and low humidity. Heavily damaged soybeans (broken or partially dehulled seeds or seeds with cuts) were rejected but used in one series of experiments (see Fig. 3). The seeds were dehulled by being broken in a mill, followed by careful separation of the hulls and crushed cotyledons. The seed coats were cleaned by sonication (Brown sonicator, 50 W, 15 min) in distilled water, manually separated from remaining seed debris, washed, and dried at 45°C under vacuum.

Microorganisms and media. Stock cultures of *A. flavus* Link ex Fries CMI 120920 and *A. parasiticus* Speare CMI 242695 were kept on potato dextrose agar. An inoculum was prepared by flooding 3- to 5-week-old cultures with sterile 0.01% Triton X-100. Spores were counted with a hematocytometer and suitably diluted with sterile 0.01% Triton X-100.

A yeast extract-sucrose (YES) broth (a modification of that described in reference 6) containing, per liter of distilled water, 200 g of sucrose, 20 g of yeast extract, 0.5 g of KH_2PO_4, 0.65 g of $K_2HPO_4 \cdot 3H_2O$, and 0.5 g of $MgSO_4 \cdot 7H_2O$ and a defined growth medium (a modification of that described in reference 21) containing 40 g of sucrose, 2 g of NH_4NO_3, 1.8 g of KH_2PO_4, 1.79 g of $K_2HPO_4 \cdot 3H_2O$, 1.5 g of $MgSO_4 \cdot 7H_2O$, and 16.7 mg of $CaCl_2$ per liter were used. Potato dextrose broth was prepared from 400 g of peeled potatoes and 30 g of glucose per liter of distilled water. A soybean-based medium consisted of 0.17 g of soybean flour per ml of 5 mM Sørensen phosphate buffer (pH 6.8).

Culture techniques. SBTI in YES broth was membrane sterilized (pore size, 0.45 μm), and 1-ml portions were inoculated with 0.15 ml of a spore suspension (10^6 spores per ml) of either *A. flavus* or *A. parasiticus*. Cultures were incubated in the dark at 25°C.

The effect of zinc and phytate on aflatoxin accumulation was studied with the defined growth medium (21). Conditions found in soybeans (8) were imitated by supplementing the medium with 4.5 mM phytate or 0.15 mM zinc sulfate or

both. Inoculation of the media and subsequent incubation were as described for YES broth.

Intact soybeans or crushed cotyledons (5 g) or isolated seed coats (1 g) in Erlenmeyer flasks were surface sterilized in 3% H_2O_2 for 2 min and subsequently rinsed twice for 1 min in sterile distilled water. Unless otherwise indicated, whole seeds and crushed cotyledons were inoculated with 5×10^4 spores of *A. flavus* in 0.5 ml of 0.01% Triton X-100 and seed coats were inoculated with 10^5 spores in 1 ml. If required, surface-sterilized soybeans were autoclaved at 121°C for 30 min just prior to inoculation.

Extraction and analysis of aflatoxin. Following incubation, mycelium and medium were extracted twice with 5 ml of CH_2Cl_2. Mycelial dry weight was determined after extraction. Inoculated soybeans or seed parts were extracted by agitation in 10 ml of CH_2Cl_2 on a shaker (150 rpm) in the dark for 30 min.

Portions of extracts were chromatographed on silica gel 60 high-performance thin-layer chromatography plates (10 by 10 cm; E. Merck AG, Darmstadt, Federal Republic of Germany) with chloroform-methanol (88:12; vol/vol) as solvent (17). Samples were analyzed in a chromatogram-scanner (excitation at 366 nm) (Camag), and the aflatoxin content was determined by peak height from standard curves prepared with pure samples. Data indicate mean values and standard deviations from duplicate experiments with duplicate or triplicate samples and are expressed in micrograms of aflatoxin (sum of AFB_1, AFB_2, AFG_1, and AFG_2 unless otherwise indicated) per milliliter of inoculated medium or per gram of seed parts. The significance of the difference between data has been analyzed statistically (Student *t* test for the comparison of means from independent samples).

RESULTS

In autoclaved whole soybeans (moisture content, 27%), the aflatoxin concentration peaked approximately 10 days after inoculation with 10^4 spores of *A. flavus* per g of seeds (Table 1). In similarly inoculated raw soybeans, the aflatoxin concentration increased slowly for 15 days and began to rise more rapidly thereafter (Table 1). A lower inoculum density (10^3 spores per g of seeds) resulted in reduced aflatoxin contamination in autoclaved soybeans; however, aflatoxin concentration in raw seeds at 30 days after inoculation reached the concentration observed in autoclaved soybeans after 10 to 15 days (Table 1). Aflatoxin accumulation in raw soybeans thus was delayed.

The in vitro effect of Kunitz SBTI on aflatoxin production by *A. flavus* and *A. parasiticus* was studied in YES broth, which is better for aflatoxin production than potato dextrose broth. SBTI purchased from Boehringer slightly enhanced aflatoxin production by both strains as well as their mycelial dry weight (Fig. 1). Identical results were obtained in experiments with SBTI from Serva (data not shown).

The influence of zinc and phytate on aflatoxin production was studied by using the defined growth medium. Neither zinc nor phytate nor both of these together, added prior to autoclaving, substantially enhanced aflatoxin production (Table 2). In contrast, addition of zinc and phytate to the autoclaved basic medium increased aflatoxin production by *A. flavus* (Table 2). Although zinc is essential for significant aflatoxin production, autoclaving the zinc-containing substrate blocks zinc availability both in the presence and in the absence of phytate. Similar results were obtained with a suspension of soybean flour in 5 mM Sørensen phosphate buffer. Enhanced aflatoxin production by *A. flavus* was observed only when zinc supplements were added after the substrate was autoclaved (data not shown).

TABLE 1. Production and degradation of aflatoxin by *A. flavus* growing on raw and autoclaved whole soybeans[a]

Inoculum (spores/g of soybeans)	Treatment	Incubation (days)	Aflatoxin (μg/g)
10^3	Raw	8	2.7 ± 3.0
		15	34.7 ± 11.5
		30	42.6 ± 17.9
	Autoclaved	8	40.0 ± 23.6
		15	44.0 ± 20.4
		30	22.6 ± 6.7
10^4	Raw	5	6.7 ± 1.2
		10	8.0 ± 2.6
		15	13.3 ± 9.8
		30	44.0 ± 10.9
	Autoclaved	5	50.7 ± 28.1
		10	68.0 ± 21.1
		15	44.0 ± 3.0
		30	29.3 ± 18.2
10^5	Raw	8	5.3 ± 6.7
		15	12.0 ± 13.2
		30	30.7 ± 17.4
	Autoclaved	8	53.3 ± 1.3
		15	33.3 ± 10.1
		30	33.3 ± 8.3

[a] The moisture content of the soybeans, including the inoculum, was 27%.

The differet kinetics of toxin accumulation by *A. flavus* observed between raw and autoclaved whole soybeans were not seen when dehulled crushed soybeans were used as substrate. In this case, 5 days after inoculation (maximum aflatoxin accumulation was reached earlier than in whole seeds), the concentration of aflatoxin in raw crushed cotyledons was 217 ± 62 μg/g, which was equal to the concentration in the autoclaved substrate (202 ± 44.9 μg/g). *A. flavus* grew on isolated seed coats and produced aflatoxin in greater quantities on autoclaved seed coats (data not shown). The ratio AFB_1/AFG_1, controlled by the substrate composition, indicated the cotyledons to be the major substrate of *A. flavus* growing on whole soybeans, since the ratio was 0.4, 0.34, and 2.6 for whole seeds, crushed cotyledons, and isolated seed coats, respectively.

To investigate whether leakage of material during autoclaving of soybeans may accelerate aflatoxin production by *A. flavus*, raw and autoclaved seeds were rinsed in 3% glucose solution (an excellent promotor of aflatoxin synthesis [1]) or in soybean broth after surface sterilization. Although the latter treatment did not influence toxin production (data not shown), 3% glucose solution slightly changed aflatoxin accumulation. However, the discrepancy between raw and autoclaved soybeans persisted (Fig. 2).

On careful selection of either intact soybeans with perfectly sound seed coats or heavily damaged seeds (broken, partially dehulled) and after the usual sterilization procedure and inoculation with *A. flavus*, we found that raw damaged seeds supported vigorous mycelial growth and sporulation, while there was comparably little sporulation on intact autoclaved soybeans and even less on intact raw soybeans (Fig. 3). While the raw damaged soybeans had 31% moisture content (including the inoculum), the intact seeds only contained 27% moisture. Six days after inoculation, 60.4 ± 20.3 μg of aflatoxin per g of seeds was extracted from heavily damaged seeds. Perfectly intact raw and autoclaved seeds

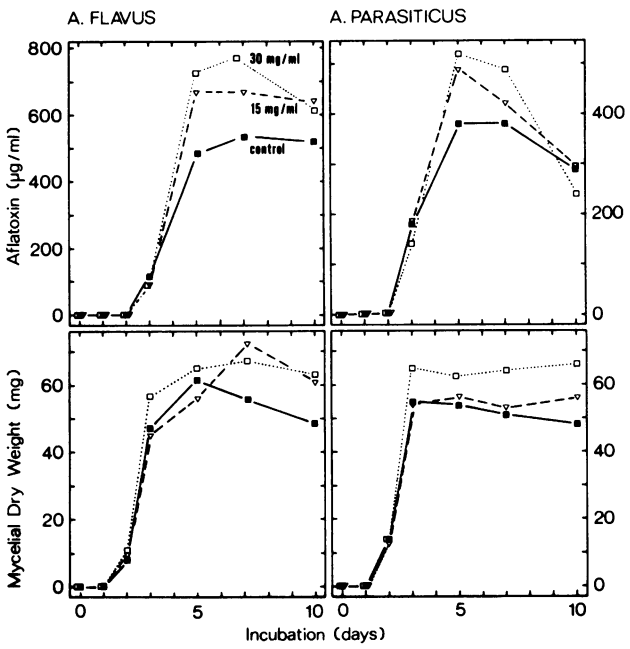

FIG. 1. Effect of Kunitz SBTI on the production of aflatoxin (AFB_1 and AFG_1) and mycelial growth of *A. flavus* and *A. parasiticus*.

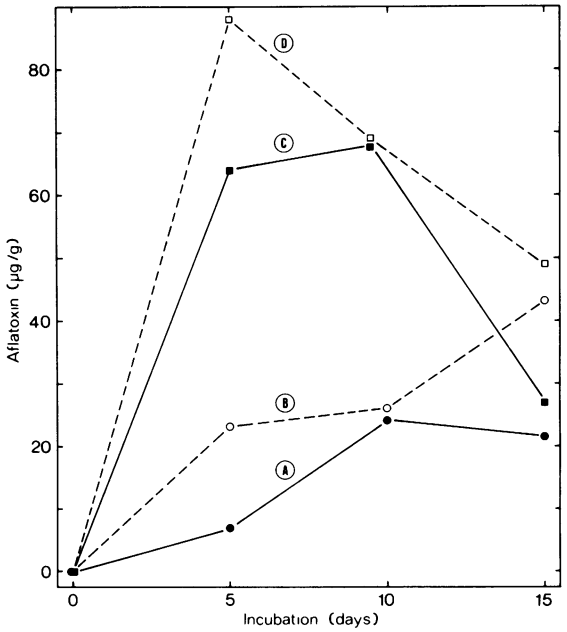

FIG. 2. Aflatoxin accumulation in whole soybeans after inoculation with *A. flavus*. After surface sterilization, the seeds were treated as follows: A, rinsed (for 2 min) in distilled water; B, rinsed in 3% glucose solution; C and D, as curves A and B, respectively, but with autoclaving.

contained significantly less aflatoxin ($P < 0.01$), i.e., 0.5 ± 0.2 and 6.4 ± 3.0 μg/g of aflatoxin, respectively.

DISCUSSION

Although *A. flavus* is among the dominating species colonizing the soybean seed surface (22), the incidence of aflatoxin contamination is low (32).

As shown in the present investigation, soybeans with 27% moisture content encourage substantial growth of and aflatoxin production by *A. flavus*. While autoclaving whole soybeans enhanced fungal growth, raw dehulled soybeans were as good a substrate as autoclaved dehulled soybeans. Accumulation of aflatoxin even surpassed the quantity of aflatoxin found in peanuts hydrated at 100% relative humidity for 5 days and incubated for 3 weeks after inoculation with *A. parasiticus* (5). Therefore, raw soybean cotyledons, the main substrate for *A. flavus* growing on soybeans, effectively promoted aflatoxin synthesis. This conclusion opposes the results in the current literature on aflatoxin accumulation in growth medium based on either raw or cooked crushed soybeans (31) and on the apparent lack of aflatoxin in unautoclaved soybean meal inoculated with an aflatoxigenic strain of *A. flavus* (16).

Heat-stable and heat-labile soybean trypsin inhibitors do not suppress the growth of *A. flavus*, since (i) soybean trypsin inhibitors did not affect aflatoxin accumulation (Fig. 1) and (ii) raw and autoclaved dehulled soybeans were just as good substrates as, e.g., hydrated peanuts (5).

Zinc, being an essential trace element for aflatoxin synthesis (21), has been used to explain the higher production of aflatoxin on autoclaved soybeans. According to Gupta and Venkitasubramanian (13), zinc is bound to phytate in raw soybeans but may be released through cooking and become more available. However, autoclaving soybean flakes and soybean protein for 30 min destroyed less than 5% of the phytate (9), and autoclaving growth media with or without phytate apparently did not improve zinc availability. This

TABLE 2. Influence of zinc and phytate on aflatoxin production by *A. flavus*

Medium[a]	Incubation (days)	Aflatoxin (μg/ml)[b]	
		AFB_1	AFG_1
Basic medium	7	5.9 ± 2.2[w]	1.9 ± 0.5[y]
	10	4.3 ± 0.3[x]	0.9 ± 0.2[z]
Zinc added before autoclaving	7	6.3 ± 2.4	1.7 ± 0.6
Phytate added before autoclaving	7	16.8 ± 7.6	8.8 ± 5.1
Zinc and phytate added before autoclaving	7	13.4 ± 8.2	3.8 ± 0.8
	10	12.1 ± 5.4	5.4 ± 1.9
Zinc and phytate added after autoclaving	7	56.8 ± 14.5[w]	42.4 ± 14.2[y]
	10	114.5 ± 34.2[x]	67.8 ± 38.2[z]

[a] The medium (21) was supplemented with 0.15 mM zinc sulfate and 4.5 mM phytate as indicated.

[b] Values followed by the same letter are significantly different ($w, x, y, P < 0.01$; $z, P < 0.05$).

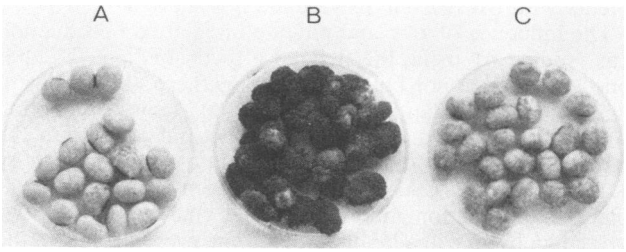

FIG. 3. Carefully selected intact (raw, A; autoclaved, C) and heavily damaged (raw, B) soybeans, respectively, 5 days after inoculation with *A. flavus*.

only improved if zinc was added after substrate autoclaving (Table 2). Therefore, cooking reduced rather than enhanced zinc availability. Moreover, the addition of zinc to autoclaved defined (Table 2) or soybean-based medium raised the ratio AFB_1/AFG_1, whereas autoclaving soybeans or their cotyledons did not. Since autoclaving apparently diminished the availability of zinc in a phytate-free medium, phytate is not the only factor affecting zinc availability.

Published data on the effect of zinc and phytate on aflatoxin production by *A. flavus* and *A. parasiticus* are contradictory because of the use of overdoses, e.g., 5 mg of zinc sulfate per g of substrate (16) (optimum concentrations are below 25 μg/ml of substrate [21]). In contrast, the slight increase in aflatoxin production in vitro through the addition of phytate (Table 2) is in agreement with published results (7).

The kinetics of aflatoxin accumulation in the various soybean substrates indicate an acceleration of aflatoxin synthesis and subsequent degradation in the order raw < autoclaved < dehulled soybeans. Simulated leakage of cotyledon constituents did not markedly accelerate aflatoxin accumulation, and degradation of antifungal seed coat constituents such as lignin (2, 12), silica (15; J. W. Krueger and F. A. Andrews, U.S. patent 3,119,805, January 1964), or hydroxyproline-rich glycoprotein (4, 10) is unlikely. However, swelling of seed coat cells and their separation from each other on cooking (30) may form cracks and pores, undoubtedly accelerating fungal colonization of the cotyledons and aflatoxin accumulation, as demonstrated by the inoculation of heavily damaged seeds with *A. flavus*.

In conclusion, it appears that seed coat integrity controls the colonization of soybeans by *A. flavus*, a barrier rendering access to the substrate difficult and ensuring low seed moisture content. Neither trypsin inhibitors nor zinc availability apparently affects seed colonization and aflatoxin accumulation.

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