Studies on Aflatoxin Detoxification in Shelled Corn by Ensiling

Lloyd A. Lindenfelser and Alex Ciegler

Aflatoxin-contaminated high-moisture corn was ensiled in an attempt to detoxify by acid-catalyzed conversion of aflatoxin B_1 to the comparatively nontoxic B_{2a}. Although moldy corn underwent a lactic fermentation, insufficient acid was produced to transform B_1.

Since the mechanical picker-sheller has come into widespread use for harvesting field corn, farmers generally are confronted with the problem of safe storage of high-moisture shelled corn. The moisture content of field-shelled corn is often too high for direct binning, and storing such grain in regu'ar farm bins without first lowering the moisture level is almost certain to result in spoilage. Molds grow readily in corn having a moisture content above 15.5%, and with growth of molds there is potential danger of mycotoxin formation (Steinberg and Nelson, 1969) which would render the grain unsuitable for feed (Forjacs, 1965; Forjacs and Carll, 1962; Hesseltine, 1967).

High-moisture corn can be preserved by ensiling (Burmeister *et al.*, 1966; Foster *et al.*, 1955). It is known, too, that aflatoxin B_1 can be converted by acid catalysis to aflatoxin B_{2a} (Ciegler and Peterson, 1968; Pohland *et al.*, 1968), a compound possessing less than $^1/_{200}$ the toxicity of B_1 (Lillehoj and Ciegler, 1969). Since lactic and other organic acids are produced in a silage fermentation (Barnett, 1954), it seemed expedient to determine whether ensiling moldy, aflatoxin-containing corn would render it nontoxic and possibly suitable for livestock feed. Similar studies have been made on the degradation of DDT in forage silage made from pasture grass contaminated with the insecticide (Henzell and Lancaster, 1969). In addition, it is possible that the mixed microflora of a silage fermentation might directly degrade aflatoxin, since it has been shown that aflatoxin partially disappears in peanuts contaminated with a mixed fungal flora (Ashworth *et al.*, 1965). However, Ashworth's study did not demonstrate that actual degradation by microorganisms was involved, and other evidence indicates that direct degradation of aflatoxin by microorganisms does not occur (Ciegler *et al.*, 1966a,b; Ciegler and Peterson, 1968).

To convert aflatoxin B_1 in moldy corn to the nontoxic form through ensiling, a lactic fermentation resulting in maximal acid production is a prime requisite. Various means have been explored for this purpose in forage silage; *e.g.*, addition of molasses or a *Lactobacillus* culture to the chopped material during silo filling (Barnett, 1954). We have tested some of these methods for their capability to enhance lactic fermentation and, subsequently, to detoxify aflatoxin in shelled corn.

MATERIALS AND METHODS

Picker-sheller, field-harvested corn was used in most of the work, although some studies were made with "elevator run," dried corn because of its ready availability. The grain was tempered to 30% moisture by adding the required amount of water and tumbling in a PK Twin Shell Blendor for 24 hr.

Fermentative production of aflatoxin-containing corn for the silo experiments was conducted in shaken culture in 2800-ml Fernbach flasks. The flasks, each containing 300 g of tempered, nonsterile corn, were inoculated with 5 ml of *Aspergillus parasiticus* NRRL 2999 aqueous spore suspension, and agitated 7 days at 28° C on a Gump rotary shaker. Sufficient water was added daily to compensate for evaporation loss. To ensure uniformity of material for the series of experiments, several lots of the mold-fermented corn were blended and kept in frozen storage for use as needed.

Preliminary experiments were conducted in 1- and 2-l. graduated cylinders as laboratory-scale, simulated silos. Additional experiments were conducted in larger laboratory silos improvised from 10 in. × 36 in. vitreous clay bell tile, designed to simulate, as nearly as possible, conditions in a regular farm silo.

In filling both graduated cylinders and tiles, the corn was added in small increments and compacted thoroughly with a wooden tamper after each succeeding addition. Compacting was necessary with the laboratory-scale silos to exclude air and prevent subsequent mold growth throughout the contents, although such precautions are not required in regular, full-scale silos (Burmeister, 1966). Incubation was at 23 to 25° C.

No opening of silos for sampling was attempted during the ensiling period because of the danger of introducing air and generally disrupting the fermentation. Instead, an adequate number of silos were always set up so that one entire unit could be sacrificed for each sample required. In opening the silos, the usual shallow surface layer of moldy grain first was taken off, then the remaining contents were removed and thoroughly mixed for a representative sample.

All aflatoxin analyses were run in duplicate. For each silo, 40-g portions of the blended contents were weighed out and extracted by a modification of the procedure of Eppley *et al.* (1968). The corn, distilled water, diatomaceous earth, and $CHCl_3$ were placed in an explosion-proof Waring Blendor and blended for 8 min, then transferred to a Büchner funnel and filtered with light vacuum. To reduce water content of the filtrate, about 20 g of anhydrous Na_2SO_4 were added with shaking, and then filtered through fluted filter paper. The filtrate was concentrated to 100 ml and processed by the column cleanup method of Eppley (1968), using double portions of all reagents and following as far as the procedure for "Fraction 2." The aflatoxin eluate was concentrated to dryness and taken up in 2 ml of $CHCl_3$ for thin-layer chromatography on silica gel plates (MN-Silica Gel G-HR, distributed by Brinkmann Instruments Co., Westbury, N.Y.). Plates were developed with the solvent system of Stubblefield *et al.* (1969); *i.e.*, water-acetone-$CHCl_3$ (1.5:12:88, v/v/v). The aflatoxin B_1 content was quantitatively analyzed by comparing the fluorescence of the unknowns spotted in different amounts with the fluorescence of an aflatoxin B_1 standard solution.

Other tests run on the ensiled corn were pH, titratable acid-

Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill. 61604

ity, and lactic acid bacteria counts. These tests were all conducted on the supernatant liquor of each sample after vigorously shaking 25 g of the corn in 225 ml of water (1 to 10 dilution, w/v) for 30 sec. The pH was obtained directly from the liquid. Acidity was determined by titrating 10-ml portions of the supernatant liquor with 0.1*N* NaOH; results were calculated and recorded as the amount of NaOH required to neutralize the aqueous extract from 1 g of corn. For the bacterial counts, the aqueous extracts were diluted serially in water, and 1-ml aliquots of the dilutions incorporated in APT agar (Baltimore Biological Laboratory) containing bromcresol purple for detecting acid-producers, and 100 μg per ml of cycloheximide to suppress mold growth. All dilutions were plated in duplicate and incubation was at 30° C. Preliminary counts were made after 1 or 2 days and final counts on the third day.

Preliminary Experiments. To establish conditions for optimal acid production during the ensiling fermentation, some of the practices used in making forage silage were tried. This work was conducted in graduated cylinders, preparatory to the experiments in the larger tile silos.

Molasses Addition. As a carbohydrate substrate for the lactobacilli in forage silage, molasses has been the most common additive (Barnett, 1954). We supplemented aflatoxin-containing corn with Brer Rabbit brand table molasses, in amounts of 1, 2.5, and 5%. Sufficient water was always added to maintain a uniform total liquid volume. The corn, molasses, and water were thoroughly mixed to ensure a uniform blend before placing into the graduated cylinders.

Bacterial Culture Addition. Normally, adequate lactic acid bacteria are present on most vegetation to initiate a forage silage fermentation (Barnett, 1954), although on some plant surfaces the numbers are frequently low (Mundt and Hammer, 1968). To give the advantage to desirable bacteria in the lactic fermentation of corn, several kinds of liquid culture were added: a broth culture of *Lactobacillus plantarum* NRRL B-531; a mixture of *L. plantarum* and *Streptococcus faecalis* NRRL B-537 broth cultures, as advocated by Whittenbury *et al.* (1967); and a bacterial mixture of predominantly acid formers, water-washed from kenaf silage (25 g of silage in 225 ml of water). Each preparation was added to the corn at the rate of 5 ml per 100 g and mixed thoroughly before placing into the graduated cylinders.

Time Studies on Acidity and pH Changes. With most forage silages the pH range is usually between 4 and 5, whether made with or without the addition of molasses (Barnett, 1954). With ensiled high-moisture corn, however, the pH may vary considerably, from highly acidic to alkaline (Burmeister *et al.*, 1966). In corn that has previously undergone a mold fermentation, pH and acidity changes that take place during the ensiling period are of especial interest. For making these observations, a series was set up of 14 graduated-cylinder silos. The graduates were filled with *A. parasiticus*-fermented, aflatoxin-containing corn, half of which was inoculated with a mixture of *L. plantarum* and *S. faecalis* broth cultures, and half was left uninoculated. At approximately 5-day intervals the contents of one each of the inoculated and uninoculated silos were removed and representative samples analyzed for pH, titratable acidity, aflatoxin B_1 content, and microbial population. Two of the graduates were reserved for a prolonged incubation of 50 days.

Additional Experiments. Studies were also carried out in larger, improvised laboratory-scale tile silos, under some of the conditions that had been established through the experiments in graduated cylinders. Still other studies were made to determine the effectiveness of acids when applied directly to affected grain for inactivating the innate aflatoxin B_1.

Corn Dilution Studies in Larger Laboratory Silos. The larger silos having a capacity of approximately 1-$^1/_2$ bushels were improvised from vitreous clay bell tile, with ceramic closure discs sealed into the bell ends as bottoms. These receptacles were filled with the aflatoxin-containing grain that had been diluted with good quality corn tempered to 30% moisture. Dilutions were 1 to 25 (4%) and 1 to 100 (1%) of aflatoxin-containing (6 μg/kg of B_1) and normal corn, respectively. Half of each of the two lots was inoculated with a mixture of *L. plantarum* and *S. faecalis* broth cultures, and the remaining two lots were left uninoculated. Each lot was thoroughly mixed before placing into its respective silo. Tops were covered and sealed with Saran wrap and rubber dam, secured with heavy rubber bands. Incubation was at room temperature for 2-$^1/_2$ mo.

At the end of incubation, contents of the silos were removed; from 2 to 3 in. of moldy grain at the tops was discarded. The remaining contents were divided roughly into halves—tops and bottoms—and each placed in frozen storage in separate plastic bags for later analyses.

Direct Acid Treatment for Aflatoxin B_1 Inactivation. It has been suggested that aflatoxin-contaminated feedstuffs might be detoxified through acid treatment (Dutton and Heathcote, 1968; Lillehoj and Ciegler, 1969). Direct acidification of contaminated corn would have the advantage of supplying hydrogen ion more quickly and in higher levels than can be achieved by ensiling. To determine the potential of direct acidification, *A. parasiticus*-fermented corn containing 6 μg/kg aflatoxin B_1 was treated with two concentrations each of HCl and lactic acid and sampled at intervals for toxin determination.

About 300 of *A. parasiticus*-fermented corn was momentarily covered with either HCl or lactic acid and immediately transferred to a large funnel lined with Masslin cloth. The concentrations of the two acids were 0.1 and 1.0*M*, and the amount retained by the corn was approximately 10% w/v. Each lot of treated corn was transferred to a 1-l. Erlenmeyer flask, and the atmosphere flushed with nitrogen gas to displace air and prevent mold growth. The flasks were stoppered and allowed to stand at room temperature. Each was sampled at intervals through 21 days and the samples were analyzed for aflatoxin content.

To determine the effect of the two acids on aflatoxin B_1 without possible interference from the corn, a parallel experiment was conducted. Pure aflatoxin B_1 was treated with several concentrations of the acids and tested at intervals for inactivation. The aflatoxin in $CHCl_3$ solution was added to the acids and allowed to stand at room temperature. Samples were taken at intervals through 71 days and each was immediately analyzed by tlc to determine the amount of B_1 that had been converted to the low R_f compound, B_{2a}.

RESULTS AND DISCUSSION

Experiments in 1-l. graduated-cylinder simulated silos indicated that, under appropriate conditions, moldy shelled corn underwent a lactic fermentation similar to that of normal high-moisture corn or of forage silage. A primary requisite for ensiling shelled corn, as with forage silage, was thorough compaction during filling to exclude air, which restricts mold growth but allows proliferation of the desirable microaerophilic acid-forming bacteria. The pH, titratable acidity, and population of acid-forming bacteria were comparable whether

Table I. Ensiling Two Blends of Contaminated Shelled Corn in "Tile Silos" (1-1/2 bushel capacity)

Silo No.	% Contaminated Corn[a]	Bacterial Inoculum Addition[b]	Sector of Silo	Acid-Formers Per G	Titratable Acidity[c]	pH	% Aflatoxin Remaining
1	4	−	Top	67×10^4	0.5	4.4	75
			Bottom	43×10^4	0.9	4.4	100
2	1	−	Top	39×10^6	0.4	4.4	70
			Bottom	87×10^5	0.7	4.6	100
3	4	+	Top	66×10^3	0.4	4.3	100
			Bottom	25×10^3	0.8	4.4	100
4	1	+	Top	93×10^2	0.5	4.3	100
			Bottom	24×10^2	0.8	4.4	100

[a] Concentration of aflatoxin B_1 in contaminated corn was 6 μg/kg. [b] Mixture of *Lactobacillus plantarum* and *Streptococcus faecalis* 24-hr broth cultures (1 to 1), added to corn at rate of 5% v/w. [c] Milliliters of 0.1*N* NaOH to neutralize the acid from 1 g of ensiled corn.

or not molasses and/or *Lactobacillus* culture was added. After a 26-day ensiling period, the pH was approximately 4, while the titratable acidity ranged between 2.2 and 2.5 ml of 0.1*N* NaOH per g of corn. Counts of acid-forming bacteria ranged from 10^4 to 10^8 per g of corn. The rapid fermentation attained acid-forming bacteria populations of 10^8 and pH of 4.3 to 4.5 in 5-day samples. As may be seen from Figure 1, the uninoculated corn actually yielded a slightly higher titratable acidity throughout the ensiling period than that inoculated with a mixture of *L. plantarum* and *S. faecalis*, and of the several types of bacterial inocula tried, little difference was seen in the effects during or at the end of the fermentation. Results were generally quite similar in the larger, 1-1/2 bushel tile silos, although the titratable acidity and populations of lactic acid bacteria were somewhat lower. The experiments conducted in tile silos are summarized in Table I.

There was little or no reduction in aflatoxin B_1 content in corn ensiled in either the graduated-cylinder or the tile-simulated silos. Correspondingly, there was little or no perceptible formation of B_{2a}. However, with direct acidification of pure aflatoxin B_1 when sufficient acid was supplied, there was complete conversion to the comparatively nontoxic form. When pure aflatoxin B_1 was subjected to an amount of lactic acid equivalent to the usual titratable acidity of ensiled shelled corn (2.5 ml. of 0.1*M*, see Figure 1), conversion to B_{2a} was complete in 21 days (Table II). In moldy corn, however, aflatoxin B_1 reacted more slowly, and when subjected to the same amount of lactic acid, full conversion was not reached in 21 days (Table III). HCl reacts much more rapidly than lactic acid with either the pure toxin or that present in contaminated corn (Tables II and III).

The amount of acidity we obtained through the lactic fermentation of corn is insufficient for effecting detoxification. This condition may be due in part to the leveling off and gradual decline in *Lactobacillus* population occurring in late phases of the fermentation. The same situation apparently occurs in forage silage, as Barnett (1954) noted that the pH in such silage does not often drop below 4. This level he attributed to a loss of acidity, possibly caused by the presence of hydrogen acceptors such as pigments. Whittenbury *et al.* (1967) considered the cause to be an inherent buffering capacity of silage, accounting for the need of increased amounts of acid to lower the pH.

Corn kernels previously attacked by mold apparently continue to deteriorate during ensiling. Individually affected kernels in final samples were brown to dark brown, or gray in color, and a musty odor was usually detectable above the typical silagelike odor of the samples. It is concluded that although ensiling is a practical means for preserving good quality, high-moisture corn on the farm, moldy, aflatoxin-containing corn is insufficiently detoxified through the process to make it safe or suitable for livestock feed.

Table II. Time Required for Acid Conversion of Pure Aflatoxin B_1 to B_{2a}

Molarity	Days Required With Acid[a] Lactic	HCl
1.0	7	0.25
0.1	21	1
0.01	54	2
0.001	71	30

[a] Concentration of aflatoxin B_1 in acid was 4 μg/ml.

Table III. Acid Inactivation of Aflatoxin B_1 in Contaminated Shelled Corn[a]

Treatment, Days	% Aflatoxin B_1 Remaining After Treatment With Acid of Molarity: Lactic 1.0	Lactic 0.1	HCl 1.0	HCl 0.1
1	100	100	33	100
7	100	100	20	100
10	100	100	16	100
14	66	100	10	66
21	50	100	4	40

[a] Concentration of aflatoxin B_1 in corn was 6 μg/kg.

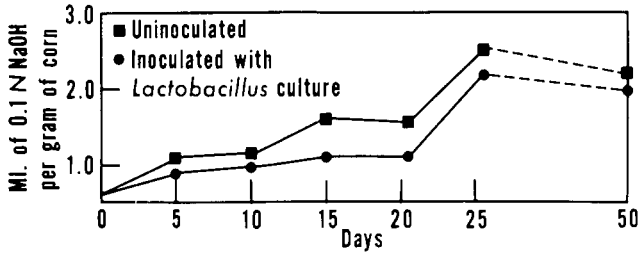

Figure 1. Changes in titratable acidity during lactic fermentation of aflatoxin-containing shelled corn in laboratory-scale graduated-cylinder silos

ACKNOWLEDGMENT

We thank Marion L. Goulden and R. R. Montgomery for valuable suggestions and assistance, and R. D. Stubblefield for the aflatoxin standards used in this work.

LITERATURE CITED

Ashworth, L. J., Jr., Schroeder, H. W., Langley, B. C., *Science* **148,** 1228 (1965).
Barnett, A. J. G., "Silage Fermentation," pp. 99–118, Academic Press, Inc.. London, 1954.
Burmeister, H. R., Hartman, P. A., Saul, R. A., *Appl. Microbiol.* **14,** 31 (1966).
Ciegler, A., Lillehoj, E. B., Peterson, R. E., Hall, H. H., *Appl. Microbiol.* **14,** 934 (1966a).
Ciegler, A., Peterson, R. E., *Appl. Microbiol.* **16,** 665 (1968).
Ciegler, A., Peterson, R. E., Lagoda, A. A., Hall, H. H., *Appl. Microbiol.* **14,** 826 (1966b).
Dutton, M. F., Heathcote, J. G., *Chem. Ind.* (London) **418,** 1968.
Eppley, R. M., *J. Ass. Offic. Anal. Chem.* **51,** 74 (1968).
Eppley, R. M., Stoloff, L., Campbell, A. D., *J. Ass. Offic. Anal. Chem.* **51,** 67 (1968).
Forjacs, J., in "Mycotoxins in Foodstuffs," G. N. Wogan, Ed., pp. 93–99, M.I.T. Press, Cambridge, Mass., 1965.
Forjacs, J., Carll, W. T., *Advan. Vet. Sci.* 7, 273 (1962).
Foster, G. H., Kaler, H. A., Whistler, R. L., J. AGR. FOOD CHEM. **3,** 682 (1955).
Henzell, R. F., Lancaster, R. J., *J. Sci. Food Agr.* **20,** 599 (1969).
Hesseltine, C. W., *Health Lab. Sci.* **4,** 222 (1967).
Lillehoj, E. B., Ciegler, A., *Appl. Microbiol.* **17,** 516 (1969).
Mundt, J. O., Hammer, J. L., *Appl. Microbiol.* **16,** 1326 (1968).
Pohland, A. E., Cushmac, M. E., Andrellos, P. J., *J. Ass. Offic. Anal. Chem.* **51,** 907 (1968).
Steinberg, M. P., Nelson, A. I., *Ill. Res.* **11,** 5 (1969).
Stubblefield, R. D., Shannon, G. M., Shotwell, O. L., *J. Ass. Offic. Anal. Chem.* **52,** 669 (1969).
Whittenbury, R., McDonald, P., Bryan-Jones, D. G., *J. Sci. Food Agr.* **18,** 441 (1967).

Received for review March 30, 1970. Accepted May 22, 1970. Work done at a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned. Presented at the American Society for Microbiology, Boston, Mass., April 1970.