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Carryover of Aflatoxin B_1 in Contaminated Substrate Corn into Nigerian Native Beer

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Aflatoxins, the toxic secondary metabolites of Aspergillus flavus and Asp. parasiticus, constitute a serious food contamination problem in Nigeria (Nwokolo and Okonkwo, 1978; Bababunmi et al 1978) and have been detected in the blood of healthy rural blood donors (Onyemelukwe and Ogbadu, 1981) and primary liver cancer patients (Onyemelukwe et al 1982) from the Guinea savannah region where traditionally brewed cereal beer is popular. A recent survey of traditional breweries in the Jos metropolis has shown a high incidence of aflatoxin B_1 contamination of their products (Okoye and Ekpenyong, 1984). The purpose of this study was to assess the efficiency of the traditional brewing in destroying aflatoxins in mould-infected substrate grains.

MATERIALS AND METHODS

Samples of red guinea corn were purchased from markets in the metropolis. Toxigenic Aspergillus flavus SA586 was obtained from the Department of Crop Protection, Institute of Agricultural Research, Ahmadu Bello University, Zaria, Nigeria. The thrombotest reagent (Nyegaard and Co., Oslo, Norway) was donated by Dr. E.O. Ayalogu, University of Port Harcourt, Nigeria. Male weanling Wistar strain albino rats were purchased from the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Jos.

Each grain sample purchased was screened for Aspergillus mould infection. A portion of each sample was moistened with sterile water and incubated at room temperature for 2 days in a beaker covered with aluminium foil. The presence of mould colonies

with morphological features similar to those of the SA586 control grown on autoclaved (0.98 kg/cm^2 for 30 min.) grains was taken as an indication of natural contamination by Aspergillus mould toxins. Samples without Aspergillus flavus-like mould growths were subsequently innoculated with grains from the SA586 control culture. 200g portions were moistened with sterile water and incubated for 5 days at room temperature in beakers covered with aluminium foil. Moisture content was controlled such that spore germination and mycelial growth occurred without grain germination.

Eight samples of suspect naturally contaminated grains and six of the artificially contaminated grain samples were used as substrates in brewing. The brewing protocol was the laboratory adaptation of the protocol of one of the traditional brewers whose products were screened in an earlier survey (Okoye and Ekpenyong, 1984). The brewer carried out the process in our laboratory, using entirely our facilities and from observing her the following fairly reproducible laboratory protocol was developed.

200-250g red guinea corn was soaked for 8 hr and then, after decanting the water, incubated for 3 days at room temperature in a beaker covered with aluminium foil. Sprouted grains were washed on the 4th day and homogenised for 60 sec at maximum speed in an MSE table homogeniser in half their volume of water. The homogenate was stirred in an aluminium pot with twice its volume of water and allowed to sediment. The sediment was heated gently, with occasional stirring, for 20 min on a bunsen burner with a previously calibrated gas flow rate, and then immediately mixed with the homogenate supernatant and allowed to cool, after which the pot was covered. On the morning of the 5th day, the hydrolysate was strained through a fine seive (diameter, ca 0.3mm) into a clean aluminium pot. The chaff was retained for aflatoxin analysis. The filtrate was heated gently (to gentle boiling) for 25 min on a bunsen burner with previously calibrated gas flow rate. On cooling, hydrolysate was decanted into an activated clay pot and allowed to ferment overnight at room temperature. The resultant liquor was allowed to mature for another 24 hr. To activate a clay pot, foam from a freshly fermenting liquor was added to a clean pot on day 3 and decanted on the 5th day; the pot was used without washing.

Alcohol content of the final product was determined by the AOAC official specific gravity method for wines (AOAC, 1975). Aflatoxins were extracted from the sprouted grain homogenate, chaff and liquor fractions by a slight modification of an earlier procedure (Okoye and Ekpenyong, 1984). Duplicate 250 ml aliquots of the liquor and 100g of homogenate fractions and whole chaff fraction were each extracted thrice with chloroform, using essentially the same sample:solvent ratio as in the earlier study. The first extraction entailed shaking for 2 hr at 100 rev/min followed by 24hr incubation of the mixture at room temperature; in the second extraction, each fraction was shaken with fresh solvent for 10 min at 120 rev/min. The third extraction was manual. Extracts of each fraction were pooled and concentrated in vacuo.

Crude aflatoxin was isolated (as aflatoxins B and G) by thin layer chromatography (TLC) in chloroform:methanol, 97:3 v/v and subjected to preliminary purification by a minicolumn adaptation of the column chromatography purification procedure of Howell and Taylor (1981). Aflatoxin B was eluted from the minicolumn with 5ml of 3% methanol in chloroform after washing with 10ml toluene followed by 3ml ether:n-hexane, 3:1 v/v. The eluate was concentrated in vacuo and further purified by two-dimensional TLC with chloroform:acetone, 90:10 v/v and chloroform:ethyl acetate:methanol, 75:20:5 v/v/v respectively, as developing solvent systems. Aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) were recovered from their respective silica gel spots by the minicolumn purification procedure. The identity of AFB_1 isolated from fractions obtained from naturally contaminated grains was confirmed by the H_2SO_4/methanol spray test (Romer, 1973). AFB_1 content of each fraction was estimated spectrophotometrically as described earlier (Okoye and Ekpenyong, 1984). In the absence of a quantitative AFB_2 standard, AFB_2 content was estimated by comparing the absorbance of its chloroform solution at 363nm with that of an AFB_1 standard (Makor Chemicals, Jerusalem). AFB_1 isolates from the liquor and starting mixture fractions were pooled separately and used for activity test.

AFB_1 activity was assayed by determining the blood clotting time and serum glucose level 3hr after intraperitoneal administration of the toxin to male rats. Three groups (given liquor AFB_1, starting AFB_1, and DMSO) of three rats each were used for each assay.

For anticoagulant activity assay, 0.044mg AFB_1/kg body weight dissolved in minimum volume of dimethylsulphoxide (DMSO) was given; the controls received the same volume of pure DMSO. Blood was collected from the eye vein using the routine technique of the Parasitology Division of NITR, Vom, and following the blood collection procedure 4b outlined in the technical manual accompanying the thrombotest reagent. The clotting time was also determined as outlined for venous blood in the manual. For the serum glucose determination, the dose was 0.02mg AFB_1/kg dissolved in minimum volume of DMSO. Blood collection and serum preparation were as described earlier (Okoye, 1982). Serum glucose level determination was by the glucose oxidase method of Wootton (1974).

RESULTS AND DISCUSSION

Mean alcohol level of the beverage (burukutu) was 3.22% by volume (2.56% by weight), a level which falls within the range of values observed for commercial burukutu in a recent survey of traditional breweries in the Jos metropolis (Onwuliri, 1984).

Of the 8 suspect naturally contaminated grain samples, AFB_1 was detected qualitatively only in two and quantitatively in one sample with carryover of 53.17 and 16.57%, respectively, into the liquor and chaff fractions. More data are needed for a meaningful deduction.

The results obtained with artificially contaminated grains are summarised in Tables 1 and 2 below.

Table 1. Aflatoxin B_1 Levels at Different Brewing Stages*

Fractions	AFB_1 Concentration (μg/fermentation mixture)	Carry over (% starting mixture AFB_1)
Starting mixture	14.92 ± 13.09	-
Solid residue (chaff)	1.49 ± 0.67	13.56 ± 5.93
Final product	5.73 ± 4.44	41.38 ± 6.48

*Each value represents mean ± S.D. of six brewing runs.

Table 2. Effect of Aflatoxin B_1 Isolates on Rat Blood Clotting Time and Serum Glucose

	Anticoagulant Activity		Hyperglycaemic Activity	
	Thrombotest time(sec)	% Increase	mg glucose/100ml serum	% Increase
Starting Mixture AFB_1	47.78±3.95(9)	72.86± 4.28	123.74±12.73(8)**	19.89±12.33
Final Product AFB_1	47.69±6.80(10)	72.54±24.78	111.64± 7.14(8)	8.17± 6.22
Control (DMSO)	27.64±0.99(7)	-	103.21±12.52(8)	-

**Significantly different from the control ($p<0.01$) as determined by the students' t test. Number of replicates in brackets.

An average of 45.06% of the initial AFB_1 concentration was completely destroyed or converted to a metabolite (or metabolites) that was sufficiently structurally modified as to behave differently from AFB_1 in the analytical procedure used. An additional 13.56% was removed in the discarded solid residue. These data suggest that the traditional brewing procedure destroys or removes a substantial fraction of the AFB_1 in contaminated substrate grain, a finding at variance with those of Chu et al (1975) and Lillehoj et al (1979) on conventional brewing. The degree of carry over (41.38%) observed in this study is comparable to the 35% recovery of added AFB_1 reported by Dam et al (1977).

AFB_1 concentration in the malt homogenate and not unsprouted grains was used as the initial concentration because of renewed fungal growth and toxin production during malting and difficulty of selecting good representative aliquots for analysis owing to non-uniform mould infection of grains in a sample. The relatively wide interexperimental variations in the initial AFB_1 concentration (Table 1) are reflections of the varying degrees of infection of the substrate grain samples used.

Liquor fraction AFB_1 isolate and AFB_1 isolated from the starting mixture caused essentially identical increases in blood clotting (thrombotest) time of the rat (Table 2), suggesting that the coumarin function of the AFB_1 molecule was not significantly modified by brewing. However, the increase in serum glucose level induced by the final product AFB_1 was significantly lower ($p<0.01$) than that induced by the same dose of starting mixture AFB_1; serum glucose level of rats given the liquor fraction AFB_1 was not significantly different ($p>0.05$) from the control. These observations suggest that the strain induced in the AFB_1 molecule by the brewing process may have significantly affected the activity of the molecular determinant of the hyperglycaemic property of AFB_1.

Owing to its relatively low level in the starting mixtures, AFB_2 was consistently detected in the other two fractions only in three of the six brewing runs, with mean levels in the starting mixture and liquor fractions of 2.48 ± 1.67 and 1.25 ± 0.79 μg AFB_2/fermentation mixture, respectively. The mean carry over into the final product was 45.59 ± 4.32%.

It appears that for a more consistent detection of AFB_2 in the chaff and liquor fractions the substrate grain samples may have to be more heavily infected. Aflatoxin G_1 was consistently detected in all the fractions but, for technical reasons, could not be quantitated.

Based on the data presented here, traditional brewing process appears to destroy or remove a substantial fraction of aflatoxins B_1 and B_2 in contaminated substrate grain. The AFB_1 carried over to the finished beer was equally substantial and biologically active, suggesting that native beer drinkers may face additional health hazards.

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