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Synthesis and Degradation of Aflatoxins by *Aspergillus parasiticus*. II.* Comparative Toxicity and Mutagenicity of Aflatoxin B_1 and its Autolytic Breakdown Products

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Abstract

A crude mycelial protein extract from a 16-day-old culture of *A. parasiticus*, on purification, lost 50% of its ability to degrade aflatoxin B_1. The addition of hydrogen peroxide increased this activity to 97% of that of the crude extract.

Ducklings dosed orally with aflatoxin extracts from 14- and 20-day-old cultures containing 46 μg or more of aflatoxin B_1 developed enlarged livers, haemorrhaged and died in less than 10 days, giving an LD_{50} of 17·5 and 17·1 μg aflatoxin B_1 per 50 g body weight respectively for each extract. When pure aflatoxin B_1 was mixed with either the crude or purified mycelial protein extract the aflatoxin B_1 level was decreased by 29% as was the toxicity of the mixture.

The main breakdown product of aflatoxin B_1 was isolated and was shown to have an R_F value of 0·34, was non-fluorescent, and was non-toxic for ducklings at oral doses as high as 400 μg per 50 g body weight.

The mutagenic effect of aflatoxin B_1 on *Salmonella typhimurium* was relative to its concentration. The main breakdown product of aflatoxin B_1 was non-mutagenic.

Extra keywords: *Aspergillus flavus*.

Introduction

Aflatoxins, such as synthesized by *Aspergillus flavus* and *A. parasiticus*, are toxic to many domestic and laboratory animals, and possibly to humans (Tung and Ling 1968; Newberne and Butler 1969; Keyl and Booth 1971; Lynch *et al.* 1972; Bryden *et al.* 1980). They are known to be carcinogenic to the rat, ferret, pig, duck and trout (Newberne *et al.* 1964; Halver 1969; Shalkop and Armbrecht 1974; Ong 1975; Wogan 1973, 1975). In culture, maximum yields of aflatoxins are obtained after approximately 14 days of incubation and then are degraded by intramycelial protein-like substances (Huynh and Lloyd 1984). Schmidt *et al.* (1977) reported the presence of peroxidase in some cultures of isolates of *A. flavus* and *A. parasiticus* and several investigators, e.g. Fishback and Campbell (1965) and Natarajan *et al.* (1975), have demonstrated that aflatoxins are effectively detoxified by hydrogen peroxide and sodium hypochlorite.

The purpose of the work reported here was to characterize the protein extract from *A. parasiticus* mycelia which could degrade aflatoxins (see Huynh and Lloyd 1984), the involvement of peroxidase, if any, and the relationship between the decline in aflatoxin levels in culture and the change in toxicity and mutagenicity.

Materials and Methods

Degradation of Aflatoxin B_1 by Extracted Mycelial Protein

Aspergillus parasiticus NRRL 2999 was grown in yeast extract (2%)–sucrose (20%) broth (YES) for 16 days, and mycelia were ground in 0·067 M phosphate buffer (pH 6·5) in the proportion of

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1 : 2 (w/v) and then mycelial protein precipitated with $(NH_4)_2SO_4$ at 80–100% saturation, the pH being maintained at 5·9 with 1 M acetic acid. Precipitated protein was harvested by centrifugation at 17000 *g* for 15 min at 5°C, and resuspended in 0·067 M phosphate buffer–saline (PBS) at pH 6·5 to regain the initial volume, and then dialysed overnight at 5°C against several changes of PBS.

Four series of sterile reaction mixtures, each four separate bottles, were made by mixing 7·45 ml of aflatoxin B_1 (100 μg/ml) in PBS with either 2 ml of mycelial extract and 0·55 ml of PBS; 2 ml of dialysed protein and 0·55 ml of PBS; 2 ml of dialysed protein, 0·5 ml of PBS and 0·05 ml of H_2O_2 (1 mM); or 2·5 ml of PBS and 0·05 ml of H_2O_2 (1 mM). The reaction mixtures were incubated at 28°C and aflatoxin levels determined (Nabney *et al.* 1965; Pons *et al.* 1972) at 24-h intervals for up to 72 h.

Extraction of Aflatoxin from Aged Cultures

Cultures (100 ml) of *A. parasiticus* NRRL 2999 grown in YES broth for 14 and 20 days were fragmented, extracted with 10% (v/v) methanol in chloroform, evaporated to dryness, and redissolved in propylene glycol. The products of several flasks were pooled and serially diluted 1 : 2 five times. The aflatoxin B_1 content of the initial dilutions from the 14-day and 21-day cultures were 920 and 515 μg/ml respectively.

Extraction of Aflatoxin B_1 With and Without Mycelial Protein

Mixtures of aflatoxin B_1 (2·5 mg in 39·75 ml PBS) and 0·25 ml H_2O_2 (1 mM) were mixed with either 10 ml PBS or 10 ml dialysed mycelial protein in duplicate, and incubated at 28°C for 72 h. Each flask was then extracted and diluted as in the previous paragraph. Aflatoxin B_1 content of the initial dilution with and without mycelial protein was 712 and 996 μg/ml respectively.

Extraction of the Main Breakdown Product (R_F 0·34) of Aflatoxin B_1

A. parasiticus cultures grown in YES medium for 16 days were treated with methanol in chloroform as before. The dried residue was laid on top of an activated aluminium oxide column (22 mm i.d. and 400 mm long) and then eluted with benzene (3 vol.)–acetone (1 vol.). The main breakdown product (R_F 0·34) was identified with fast blue B–NaOH solution (Lee *et al.* 1974) and further purified by silica gel t.l.c. The washed and dried crystal products were added together and mixed in propylene glycol at concentrations of 4, 2, 1, 0·5 and 0·25 mg/ml.

Toxicity Tests

One-day-old Peking ducklings (50–55 g liveweight) in groups of 10 were given orally (0·1 ml/bird) one of the doubling dilutions of aflatoxin from the 16- and 20-day culture extracts, or the aflatoxin B_1 with and without mycelial protein extracts. Control ducklings received 0·1 ml of propylene glycol. The ducklings were brooded together at 27°C with food and water *ad libitum*, and were weighed daily. Those ducklings which survived were killed at day 10. Livers were removed shortly after death, weighed and fixed in 4% (v/v) formaldehyde-buffered saline. Paraffin sections were stained with Erhlich's acid haematoxylin–eosin solution. The LD_{50}s were estimated by the method described by Reed and Muench (1938).

Mutagenicity Tests

The mutagenic potencies of the following dimethyl sulfoxide solutions were evaluated using the Ames test (Ames *et al.* 1975) with *Salmonella typhimurium* TA 98 as the indicator bacterium: the 14- and 20-day culture extracts of aflatoxin B_1 at concentrations of 0·05 and 0·028 μg/ml respectively; the aflatoxin B_1 with and without mycelial protein extracts at concentrations in terms of aflatoxin B_1 of 0·05 μg/ml from the mixture aflatoxin B_1 and hydrogen peroxide, 0·035 μg/ml from the mixture aflatoxin B_1, hydrogen peroxide and mycelial protein; the main breakdown product of aflatoxin B_1 with an R_F value of 0·34 at concentrations of 0·05 and 0·2 μg/ml.

Infrared Spectra of Aflatoxin B_1 and Its Main Breakdown Product

Aflatoxin B_1 or its main breakdown product (R_F 0·34) was emulsified in paraffin, a thin film of the emulsion spread between two KCl cells and an infrared spectrum obtained for each sample.

Results

Degradation of Aflatoxin B_1 by Extracted Protein

When a mixture of aflatoxin B_1 and mycelial extract was incubated for 72 h, the amount of aflatoxin B_1 decreased from 745 to 575 μg, equivalent to a rate of 7·4% per 24 h (Table 1). Aflatoxin degradation due to dialysed mycelial protein was equivalent to 3·7% per 24 h, approximately 50% of the activity of crude mycelial extract. Addition of hydrogen peroxide resulted in an increase in the degradation rate of dialysed protein to 7·2% per 24 h, which was approximately 97% of the activity of crude mycelial extract.

Table 1. Effect of mycelial extract and precipitated mycelial protein from *A. parasiticus* on degradation of aflatoxin B_1 at different incubation times

Reaction mixture	Aflatoxin B_1 (μg) after incubation for: 0 h	24 h	48 h	72 h	Aflatoxin degradation per 24 h due to mycelial extract or protein (%)[A]
Aflatoxin + mycelial extract	745	685	630	575	7·4 (100·0)[B]
Aflatoxin + dialysed mycelial protein	745	715	685	660	3·70 (49·8)
Aflatoxin + dialysed mycelial protein + H_2O_2	745	690	643	580	7·2 (97·1)
Aflatoxin + H_2O_2	745	745	740	740	0 (0)

[A] Values have been decreased by 0·16 which represents the percentage of aflatoxin degradation due to the pH of the medium.
[B] Values in parentheses are the percentage activities of the mixtures relative to the mycelial extract.

Toxicity

Aflatoxin extracted from aged cultures

Aflatoxin extracted from the 14-day-old cultures was toxic to all ducklings when challenged at the two highest dose levels used (92 and 46 μg aflatoxin B_1). The ducklings were inappetent and died within 24 h (Table 2). The 20-day-old culture extracts contained considerably less aflatoxin B_1 than did the 14-day-old ones, and were less toxic. Only in the group challenged with the first dose level (51 μg of aflatoxin B_1) did all the ducklings die. Their livers were also enlarged and haemorrhagic. The LD_{50} for the 14-day-old culture extract was 17·5 μg of aflatoxin B_1 per 50 g liveweight.

Ducklings given 14-day-old culture extracts at dose levels in the range of 92–46 μg of aflatoxin B_1 lost 11–12% of their liveweight before death. The 20-day-old culture extract was less toxic; those ducklings which survived the third dose level (7 out of 10) grew at a rate similar to that of the controls.

Aflatoxin B_1 with and without mycelial protein

Addition of mycelial protein to a mixture of aflatoxin B_1 and hydrogen peroxide decreased the toxicity of the mixture by 29% (Table 2). However, the LD_{50}, expressed in terms of aflatoxin B_1 present in each mixture, remained similar (17·7 and 17·9), showing that the breakdown products of aflatoxin B_1 were non-toxic on addition of mycelial protein to the mixture.

The main breakdown product (R_F 0·34) of aflatoxin B_1

The main breakdown product of aflatoxin B_1 with an R_F value of 0·34 was non-toxic, even when ducklings were challenged with up to 400 μg per 50 g liveweight. All liver sections taken from these ducklings were devoid of haemorrhage, parenchymal necrosis and large nuclei.

Table 2. Response of ducklings to aflatoxin extracts from 14- and 20-day-old cultures of *A. parasiticus*, aflatoxin B_1 with and without mycelial protein, and a breakdown product of aflatoxin B_1 with an R_F value of 0·34

	Responses of ducklings at aflatoxin dose levels[A] of:					Response of control ducklings	LD_{50} per 50 g liveweight (μg aflatoxin B_1)[B]
	1	2	3	4	5		
14-day-old culture extract							
Mortality[C]	10	10	7	2	1	0	17·5
Haemorrhage[D]	++++	+++	++	++	0	0	
Ratio of liver weights to liveweight (%)	4·5	4·3	4·0	3·4	3·0	3·0	
Difference between initial liveweight and final liveweight (%)	−11	−12	+198	+220	+225	+224	
20-day-old culture extract							
Mortality[C]	10	7	3	1	0	0	17·0
Haemorrhage[D]	++++	++	+	+	0	0	
Ratio of liver weight to liveweight (%)	4·5	4·0	3·6	3·1	3·1	3·1	
Difference between initial liveweight and final liveweight (%)	−11	−6	+228	+230	+237	+228	
Aflatoxin B_1 + H_2O_2							
Mortality[C]	10	10	7	3	0	0	17·7
Haemorrhage[D]	++++	++++	+++	++	0	0	
Ratio of liver weight to liveweight (%)	5·4	5·3	4·4	3·7	3·4	3·4	
Difference between initial liveweight and final liveweight (%)	−12	−12	+168	+178	+192	+195	
Aflatoxin B_1 + mycelial protein + H_2O_2							
Mortality[C]	10	9	5	1	0	0	17·9
Haemorrhage[D]	++++	+++	+++	+	0	0	
Ratio of liver weight to liveweight (%)	5·4	5·2	4·0	3·4	3·4	3·4	
Difference between initial liveweight and final liveweight (%)	−11	+160	+166	+179	+175	+175	
Breakdown product of aflatoxin B_1							
Mortality[C]	0	0	0	0	0	0	
Haemorrhage[D]	0	0	0	0	0	0	
Ratio of liver weight to liveweight (%)	3·4	3·4	3·3	3·2	3·4	3·4	
Difference between initial liveweight and final liveweight (%)	+197	+200	+197	+200	+198	+198	

[A] The first dose level of the 14-day-old culture extract contained 92 μg of aflatoxin B_1 and that of the 20-day-old culture extract, 51 μg. The first dose level of aflatoxin B_1 and H_2O_2 mixture contained 99 μg of alfatoxin B_1 and the same mixture with the addition of mycelial protein, 71 μg. The first dose level of the breakdown product of aflatoxin with an R_F value of 0·34 was 400 μg. All dose levels were serially diluted by doubling the dilution 5 times.

[B] LD_{50}'s were calculated by determining the amount of aflatoxin B_1 in the mixture which was lethal to 50% of the ducklings. [C] No. of deaths per 10 ducklings. [D] Arbitrary scale.

Mutagenicity

There was a significant decrease ($P<0.01$) in the number of mutants induced by the 20-day-old relative to that of the 14-day-old culture extract (Table 3). The 20-day-old extract (containing 0·028 μg of aflatoxin B_1/ml) induced 297 mutants whereas the 14-day-old extract (containing 0·05 μg of aflatoxin B_1/ml) induced 518 mutants per 100 μl.

Addition of mycelial protein to a mixture of aflatoxin B_1 and H_2O_2 significantly reduced ($P<0.05$) the number of mutants, relative to the same mixture without mycelial protein. Aflatoxin B_1 with mycelial protein (containing 0·035 μg aflatoxin B_1/ml) induced 357 mutants, but when the mycelial protein was omitted, 480 mutants per 100 μl. The breakdown product of aflatoxin B_1 (R_F 0·34) induced few, if any, mutants (Table 3).

Table 3. Numbers of *Salmonella typhimurium* mutants, induced by extracts from 14- and 20-day-old cultures of *A. parasiticus*, aflatoxin B_1 (AFB_1) with and without mycelial protein, and a breakdown product of aflatoxin B_1 with an R_F value of 0·34

Inducers	No. of mutants[A] per plate for amounts of inducer of:			
	25 μl	50 μl	100 μl	200 μl
14-day-old culture extract (0·05 μg AFB_1/ml)	128	260	518	>1000
20-day-old culture extract (0·028 μg AFB_1/ml)	74	150	297	>500
Aflatoxin B_1 + H_2O_2 (0·05 μg AFB_1/ml)	117	230	480	>1000
Aflatoxin B_1 + H_2O_2 + mycelial protein (0·035 μg AFB_1/ml)	87	157	357	>500
Breakdown product of aflatoxin B_1 (0·05 μg/ml)	0	0	0	0
Breakdown product of aflatoxin B_1 (0·20 μg/ml)	0	0	6	12

[A] The mean number of mutants appearing on control plates was 23. Therefore the net numbers of mutants for each plate with inducer was obtained by deducting 23 from the total count.

Infrared Spectra of Aflatoxin B_1 and its Main Breakdown Product

The aflatoxin B_1 infrared spectrum showed a specific peak at 1750 cm^{-1} corresponding to the lactone ring. The peak, absent in the infrared spectrum of the R_F 0·34 non-fluorescent breakdown product, which had a new band at 3400 cm^{-1}, indicated the presence of a free hydroxyl group.

Discussion

When a number of toxigenic isolates of the *Aspergillus* group were grown in culture, maximum yields of aflatoxin occurred at about 14 days, followed by a marked decline as the cultures aged (Huynh and Lloyd 1984). This decline could be attributed to aflatoxin being degraded within the mycelium by fungal enzymes, and was supported by the observations that a mixture of precipitated protein from a mycelial extract and hydrogen peroxide could degrade aflatoxin B_1 almost as effectively as the mycelial extract alone, that when radioactive aflatoxin was degraded most of the radioactive metabolites appeared in the mycelium, and that removal of the mycelium from the culture resulted in no further decline in aflatoxin levels (Huynh and Lloyd 1984).

The precipitated protein from the mycelial extract could degrade aflatoxin B_1 alone without addition of hydrogen peroxide as a substrate. This suggested that beside peroxidase enzyme(s), at least another enzyme is involved in the degradation of aflatoxin by *A. parasiticus*. This enzymatic process is much more complex than that suggested by Doyle

and Marth (1979). They showed that a protein fraction obtained from mycelial extract of *Aspergillus* spp. by treatment with ammonium sulfate at 45% saturation in the presence of hydrogen peroxide was responsible for much, if not all, of their observed aflatoxin degradation. The degradation product of these peroxidase enzymes was aflatoxin B_{2a}, a 2-hydroxy derivative of aflatoxin B_1 whereas in our work the main breakdown product is a non-fluorescent compound with a R_F value of 0·34. Microorganisms other than *A. flavus* and *A. parasiticus* can biologically degrade aflatoxins. *A. niger*, *A. terreus*, *A. luchuensis*, *Penicillium raistrickä* (Ciegler *et al.* 1966), *Rhizopus arrhizus*, *R. stolonifer* (Cole *et al.* 1972), *Trichoderma viride* and *Mucor ambigus* (Mann and Rehn 1976) were reported to be able to transform partially aflatoxins to new fluorescing compounds by hydrogenation of the aflatoxin component but leaving the cyclopentenone moiety intact. In our work it was shown, by infrared spectral analysis of the main breakdown product, that destruction of aflatoxin was by degradation of the cyclopentenone moiety, principally the destruction of the lactone ring. This suggests aflatoxin B_1 is possibly degraded by *A. parasiticus* by different mechanism to these other organisms.

Toxicity of extracts from different aged cultures and aflatoxin mycelial protein mixtures was shown to be dependent on the aflatoxin B_1 level in each sample. A decrease in mutagenic activity was observed in the 20-day-old culture extracts, in aflatoxin mycelial protein mixtures compared with the 14-day-old culture extracts and in aflatoxin B_1 solution alone. The main breakdown product of aflatoxin B_1 was shown to be non-toxic to ducklings and non-mutagenic. This suggests that aflatoxin B_1 was degraded by *A. parasiticus* to products which were substantially less toxic and mutagenic. Lee *et al.* (1974, 1981) showed that aflatoxin D_1, obtained from cleavage of the lactone ring of aflatoxin B_1 by treatment with ammonia at elevated temperature and pressure was 94% less toxic and 99·8% less mutagenic than aflatoxin B_1. Our results suggest that a similar compound to aflatoxin D_1 is the result of natural breakdown. Hsieh *et al.* (1977) showed that hydroxylation of the cyclopentenone moiety also resulted in decreased toxicity as aflatoxin Q_1 was apparently one-eighteenth as toxic to chick embryo as aflatoxin B_1. Ciegler and Peterson (1968) demonstrated that aflatoxin B_2 had one-hundredth the toxicity to ducklings as has aflatoxin B_1, and its mutagenicity potency was zero relative to aflatoxin B_1. Buchi *et al.* (1973) showed that aflatoxin P_1, found in urine of the Rhesus monkey, with a mutagenic potency of 1 in 100, caused mortality in mice which was one-fifteenth of that caused by aflatoxin B_1. The data reported here, along with previous reports, show that there is a strong reduction in mutagenicity and toxicity of aflatoxin B_1 with alteration of any portion of the aflatoxin molecule. This suggests that the simple method of assaying aflatoxin B_1, based on extraction and easily observed fluorescence, appears valid as a reliable measure of toxicity for public health purposes, as there is a direct relationship between loss of fluorescence at R_F 0·56 and loss of toxicity under natural circumstances.

A variety of physical and chemical means such as ultraviolet irradiation, heat, aqueous solutions of strong acids and bases, and oxidizing agents have been studied as a means of detoxifying aflatoxin-contaminated foods and feeds but practical application of such treatments is doubtful because some did not effectively inactivate the toxin and some altered the chemical composition of the meals and lowered their protein efficiency ratio values. More information is needed before the usefulness of bisulfite in treating aflatoxin-contaminated products can be assessed (Marth and Doyle 1979). Now that it has been demonstrated that degradation of aflatoxin by *A. parasiticus* can occur enzymatically, the biological degradation of aflatoxin by this method may offer another possible solution to the problem.

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