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ENZYMATIC DEGRADATION OF AFLATOXINS IN SHAKE CULTURES OF *ASPERGILLUS FLAVUS* CMI 102566: A POSSIBLE ROLE OF PEROXIDASE IN BIOLOGICAL CONTROL MECHANISM

VED PAL SINGH
Applied Microbiology and Biotechnology Laboratory, Department of Botany, University of Delhi, Delhi 110 007, India.

During the early stages of growth of the toxigenic *Aspergillus flavus* CMI 102566, under shake culture conditions, the level of total aflatoxins was found to increase, showing maximum concentration in 3-day old culture. At d-4 and d-7, there was a direct correlation between decreased levels of aflatoxins and the increased activities of peroxidase enzyme in the mycelial extracts of *A. flavus*. The present findings suggested a potential role of mycelial peroxidase of *A. flavus* in the *in vivo* degradation of aflatoxins, which were otherwise produced by this toxigenic fungus itself.

Keywords: Aflatoxins; *Aspergillus flavus*; Biological control; Peroxidase.

Introduction

Aflatoxins constitute the most widely studied of all mycotoxins and their toxic, mutagenic, teratogenic as well as immunocarcinogenic effects in man and animals have been well documented[1-4]. Efficient degradation of aflatoxins in contaminated agricultural produce or the raw materials used for food and feeds could be of great significance to human and animal health[5]. The ability of the toxigenic fungi, *Aspergillus parasiticus*[6-12] and *Aspergillus flavus*[13-14] to degrade aflatoxins has been demonstrated.

Proteinanceous nature of aflatoxin-degrading factor has also been reported in the mycelia of *A. parasiticus*[10] and *A. flavus*[13]. However, peroxidase activity in the mycelia of *A. parasiticus* has been implicated in aflatoxin degradation[10]. The present study was, therefore, undertaken to investigate the role of mycelial peroxidase in the degradation of aflatoxins in *A. flavus* CMI 102566 under shake culture conditions.

Materials and Methods

Organism and culture conditions: Aspergillus flavus (Strain, CMI 102566), which was kindly supplied by Professor J.E. Smith of University of Strathclyde, Glasgow, UK, was used in the present investigation. The strain was maintained at 4°C on slants of potato dextrose agar (PDA) medium and subcultured at 30°C. The inoculum was prepared from cultures grown at 30°C for 10 days on PDA. Fungal spores were harvested with sterile wetting agent (three drops of tween – 80 suspended in 100 ml of distilled water). Suitable dilutions were made to achieve an inoculum density of $2x10^6$ spores ml^{-1}. Low glucose nutrient broth (nutrient broth with 1% glucose in distilled water) was used in all the experiments, as this medium supports maximum growth of *A. flavus* with substantial amount of aflatoxin production in shake cultures. The liquid medium (100 ml each) was dispensed into 250 ml conical flasks, plugged with non-absorbent cotton wool and autoclaved at 121° C(15 p.s.i. pressure) for 15 minutes after wrapping the plugged mouth of the flask with aluminium foil. Each flask was inoculated with $2x10^6$ spores and incubated at 30° C on an orbital shaker at 200 rpm. The cultures were harvested at selected timings (i.e., on 1st, 2nd, 3rd, 4th and 7th day of growth of *A. flavus* so as to have at least five samples spreading over a period of one week)

for aflatoxin extraction and analysis. From each batch of flask, mycelia were harvested for carrying out peroxidase assays.

Extraction and analysis of aflatoxins: Cultures were subjected to extraction of aflatoxins by a modification of the procedures of Shih and Marth[15], where whole cultures were blended using Waring blender for 2 minutes. The same extraction procedures were used for both separated mycelia and culture filtrates. After the cultures were filtered and washed, 80 ml of the filtrate was used for extraction; to this 100 ml of chloroform was added and shaken vigorously. The chloroform extract was removed and the remaining material extracted twice with 100 ml of chloroform. Pooled chloroform extracts were evaporated until a few ml of the sample was left in each case. Samples were evaporated under nitrogen in a sample concentrator at 60°C. The final dried aflatoxin samples were dissolved in 1 ml chloroform. Aliquotes (100 µl) of each sample, together with aflatoxin standards were spotted on TLC plates coated with silica gel G (Merck) and developed in toluen/ethylacetate/chloroform/90% formic acid (70:50:50:20, by volume). The plates were then dried in a fume cupboard and observed under long wave UV light (366 nm) in a dark viewing cabinet. The fluorescence of the samples with respect Rf values of standard aflatoxins B_1, B_2, G_1 and G_2 was observed; the areas marked for each aflatoxin and scraped off for quantitative estimation by the method of Nabney and Nesbitt[16], using a Shimadzu UV-120-02 spectrophotometer.

Cell-free enzyme preparation and peroxidase assays : The mycelia obtained at each stage were washed thoroughly with ice-cold distilled water and homogenised by mortar and pestle in a fixed volume (25 ml) of 0.05 M tris-HCl buffer (pH 7.0), using glasperlen (0.10 – 0.11 mm ϕ). The homogenate was centrifuged at 15,000 x g for 30 min in a MSE High Speed-18 centrifuge at 4°C. The supernatant (cell-free extract) obtained was stored under ice-cold condition and used for the analysis of peroxidase activity and protein estimation.

Table 1. Mycelial/filtrate aflatoxin concentrations (levels) in *Aspergillus flavus* CMI 102566 at different stages of growth under shake culture conditions.

Growth Period (days)	Mycelial/ filtrate	Aflatoxin concentrations (ppm/100ml culture)					
		Individual aflatoxins					
		B_1	B_2	G_1	G_2	Sub total	Grand total
1	Mycelial	ND	ND	ND	ND	ND	
	Filtrate	ND	ND	ND	ND	ND	ND
2	Mycelial	0.343	0.198	0.176	ND	0.717	
	Filtrate	0.093	0.026	0.324	ND	0.443	1.160
3	Mycelial	0.422	0.385	0.472	0.428	1.707	
	Filtrate	0.551	0.104	0.574	0.366	1.595	3.302
4	Mycelial	0.128	0.261	0.463	0.214	1.066	
	Filtrate	0.415	0.287	0.287	0.202	1.191	2.257
7	Mycelial	0.336	0.058	0.370	ND	0.764	
	Filtrate	0.336	0.320	0.166	ND	0.822	1.586

ND, Not Detectable.
The results are the means of triplicate determinations (reproducibility ± 5%)

Table 2. Mycelial peroxidase activities in the cell-free extracts of *Aspergillus flavus* CMI 102566 at different stages of growth under shake culture conditions.

Growth period (days)	Specific activities of peroxidase (AU/min/mg protein)
1	0.01056
2	0.01750
3	0.01893
4	0.11218
7	0.15962

AU, Absorbancy Units at 475 nm.
The results are the means of triplicate determinations (reproducibility ± 5%)

Peroxidase activity in the cell-free extract was assayed by the method of Polis and Shmukler[17], using dihydroxyphenyl alanine (DOPA) as hydrogen donor and hydrogen peroxide (H_2O_2) as an oxidant. DOPA reacts with peroxidase and H_2O_2 to yield a red oxidation product having maximum absorbancy at 475 nm.

Protein in the extract was estimated by the method of Lowry *et al.*[18], using bovine serum albumine (Sigma) as standard. The enzyme activity (specific activity) of peroxidase was expressed as the increase in Absorbancy Units at 475 nm due to the enzymatic oxidation of DOPA per min per mg of protein (i.e., AU/min/mg protein).

Results and Discussion

The results obtained for mycelial as well as filtrate aflatoxin levels in the cultures of *A. flavus* at different stages of growth are presented in Table 1. The data indicates that in one-day old culture, no detectable amount of aflatoxin was observed. However, there was an increase in the total concentration of aflatoxins and was found to be maximum at d-3 of the growth of *A. flavus*. But in 4- and 7- day old cultures, decreased levels of aflatoxins were noticed. There was about 31% decrease in total aflatoxin level at d-4 with a further decrease of about 30% at d-7 of growth (Table 1). However, aflatoxin G_2, both in the mycelium as well as in the culture filtrate, was found to appear only at d-3 with its decreased concentration at d-4 and its complete absence at d-7 of the growth of *A. flavus*. These observations suggest that the stage of secondary metabolism for aflatoxin biosynthesis sets in after d-1 and continues till d-3 when maximum level of aflatoxins is reached. However, at subsequent stages (d-4 and d-7) of growth of *A. flavus*, the decreased levels of aflatoxins could be because of their degradation, thereby bringing down the level of aflatoxins at d-7 to about 40% of that obtained at d-3.

Table 2 presents observations on mycelial peroxidase activities at different growth phases of *A. flavus*. The specific activities of peroxidase enzyme increased initially up to d-2 only marginally and remained almost same up to d-3. This suggests that the initial increase in peroxidase activity from d-1 to d-2, and almost constant level of enzymatic activity from d-2 to d-3 (Table 2) might be attributed to growth phase-mediated and developmentally regulated secondary metabolism; this is quite evident from the fact that one-day old mycelium of *A. flavus* exhibited peroxidase activity without showing the presence of aflatoxins in the cultures of this toxigenic fungus. From the data shown in Tables 1 and 2, it is quite clear that there appeared to be a correlation be-

tween decreased aflatoxin levels (Table 1) and increased peroxidase activities (Table 2) during d-4 and d-7 (i.e., after d-3) of the growth of *A. flavus*.

The present findings suggested the participation of mycelial peroxidase of *A. flavus* in the *in vivo* degradation of aflatoxins produced by this toxigenic fungus itself under shake culture conditions. The potential role of cell-free extracts of *A. parasiticus* (which also show peroxidase activities) in aflatoxin degradation has also been demonstrated[10]. Moreover, the role of peroxidase related activity of cytochrome P-450 in biotransformation/biodegradation of various heterocyclic xenobiotic compounds in a variety of eukaryotic systems is well documented[19-21].

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