Positive correlation exists between glutathione S-transferase activity and aflatoxin formation in *Aspergillus flavus*

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The presence of glutathione (GSH) S-transferase activity, using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate, has been established in the cytosolic fraction of the toxigenic (aflatoxin producing) and non-toxigenic strains of *Aspergillus flavus*. Significant differences in the GSH S-transferase activity were observed between the toxigenic and non-toxigenic strains. A positive correlation has been demonstrated for the first time between aflatoxin formation and a biochemical parameter, namely GSH S-transferase activity. The evidence in support of *A. flavus* GSH S-transferase induction by endogenous aflatoxins is as follows: (i) the age-related production of aflatoxin follows the same pattern as the cytosolic GSH S-transferase activity profile; (ii) significantly higher enzyme activity was associated with mycelia of a toxigenic strain grown in medium supporting high aflatoxin production (sucrose–low-salts medium) while the enzyme activity was low in medium producing less aflatoxin (glucose–ammonium nitrate medium). The GSH S-transferase activity of the non-toxigenic strain was hardly affected by a change in the medium as it produces no aflatoxins; and (iii) the toxigenic strain demonstrated significantly higher apparent $V_{\text{max}}$ with no change in $K_m$ as compared with the non-toxigenic strain. This indicates that the enzyme induction by endogenous aflatoxins is similar to the action of phenobarbital and other inducing drugs (Kaplowitz et al., 1975).

**INTRODUCTION**

Glutathione (GSH) S-transferases (EC 2.5.1.18) are a family of multifunctional proteins, largely cytosolic, non-specific enzymes catalysing the conjugation of GSH with a wide variety of xenobiotics (Boyland & Chasseaud, 1969; Jakoby, 1978). GSH S-transferase acts as a ‘triple threat’ in the detoxification and subsequent removal of noxious agents (Chasseaud, 1979). Much information is available regarding this enzyme in animal species, although it has been detected in plants, insects and micro-organisms with appreciably lower specific activity (Lau et al., 1980; Shishido, 1981; Wackett & Gibson, 1982; Cohen et al., 1986).

Study of GSH S-transferase of *Aspergillus flavus* stems from its ability to synthesise the aflatoxins, a group of mycotoxins of which aflatoxin B$_1$ (AFB$_1$) is the most potent hepatocarcinogen (Newberne & Butler, 1969; Enomoto & Saito, 1972; Wogan, 1973; Krishnamachari et al., 1975). The epoxidation of AFB$_1$ by the microsomal cytochrome P-450 system is the activation pathway (Swenson et al., 1974), while the formation of AFB$_1$:GSH catalysed by GSH S-transferase is the major activation pathway for the electrophile AFB$_1$:8,9-oxide (Degan & Neumann, 1978). Recently, the specificities of various subunits of rat liver GSH S-transferases for the GSH conjugation of putative AFB$_1$:8,9-oxide have been reported by Ketterer and co-workers (Ketterer et al., 1983; Coles et al., 1985). They have shown that AFB$_1$:8,9-oxide is a substrate for Ya- and Yc-containing GSH S-transferases. The Co-binding haemoprotein present in *Aspergillus parasiticus* (Bhatnagar et al., 1982) can play a role in the formation of AFB$_1$:8,9-oxide, which may serve as a substrate for fungal GSH S-transferase. This preliminary report deals with the activity of GSH S-transferase in toxigenic and non-toxigenic strains of *A. flavus* under different culture conditions and growth periods to correlate the production of aflatoxins with the conjugating enzyme activity. The presence of GSH S-transferase and its induction by endogenous aflatoxins has been established for the first time in this paper.

**MATERIALS AND METHODS**

*A. flavus* NRRL-3537 and NRRL-5565 (nontoxigenic) and *A. parasiticus* NRRL-3240 and NRRL-2999 (toxigenic) were obtained from Northern Regional Research Laboratory, Peoria, IL, U.S.A. The strains were maintained as soil cultures. GSH and β-mercaptoethanol were obtained from Sigma Chemical Co., St. Louis, U.S.A. 1-Chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) were the products of Aldrich Chemical Co., Milwaukee, WI, U.S.A. Other chemicals were of analytical reagent grade obtained from BDH (India) or S-Merck (India). Sodium [1-¹⁴C]acetate (specific radioactivity 58.46 mCi/mmoll) was obtained from Bhabha Atomic Research Centre, Bombay, India.

**Media composition**

GPA medium consisted of glucose 20 g, agar 20 g and peptone 10 g per litre of double-distilled water. Sucrose–low-salts medium (SLS) and glucose–ammonium nitrate

Abbreviations used: GSH, glutathione; AFB$_1$, aflatoxin B$_1$; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; SLS, sucrose–low-salts medium, GAN, glucose–ammonium nitrate.
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(GAN) medium were made up according to Gupta & Venkitasubramanian (1975) and Brian et al. (1961), respectively.

Culture conditions
The *Aspergillus* strains were initially grown in bottles containing 50 ml of GPA medium for a period of 6–7 days. Spore suspensions (1.38 × 10^7–1.45 × 10^7 spores/ml) were prepared in sterile double-distilled water and inoculated into flasks containing 100 ml of SLS medium. Flasks were incubated at 28 ± 1 °C as stationary cultures and harvested at the end of the required growth periods. The fungal mycelium was separated, washed and used for the estimation of enzyme activity.

Preparation of fungal cytosol
Mycelia was treated with liquid N_2 and then a 30% (w/v) homogenate was made by homogenizing in 10 mM-phosphate buffer, pH 7.0 (containing 0.25 mM-sucrose and 1.4 mM-β-mercaptoethanol). The homogenate was centrifuged at 12000 g for 20 min at 4 °C. The supernatant was centrifuged at 100000 g for 1 h to obtain the cytosolic fraction.

Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin (Sigma Chemical Co.) as standard. GSH S-transferase activity was assayed by the method of Habig et al. (1974). For kinetic studies, initial velocity of the reaction was measured as a function of GSH concentration and expressed in terms of nmol of CDNB conjugated/min.

Aflatoxins were extracted with chloroform and separated by thin-layer chromatography using 2% (v/v) methanol in chloroform as solvent (Gupta et al., 1975). Aflatoxin was eluted with methanol and estimated spectrophotometrically by the method of Nabney & Nesbitt (1965).

RESULTS
The growth of all strains was exponential from 48 to 96 h and remained stationary thereafter (Fig. 1). The non-toxigenic strain showed considerably higher growth than that of the toxigenic strain. The cytosolic GSH S-transferase activity using CDNB as a substrate was linear with respect to time and enzyme concentration. No activity was evident with DCNB as a substrate. The toxigenic strains were found to possess significantly higher GSH S-transferase activity than the non-toxigenic strains over the entire growth period studied (Fig. 2).

Both specific and total GSH S-transferase activities of the toxigenic strain were substantially higher when the mycelia were grown in SLS medium compared with GAN medium (Fig. 3). The GSH S-transferase activity of the non-toxigenic strain remained the same irrespective of the growth medium. The production of aflatoxins by *A. parasiticus* mycelia was ten times higher when grown in SLS medium compared with GAN medium (Table 1). Results summarized in Table 2 demonstrate significantly higher incorporation of [1-14C]acetate into aflatoxin by

Fig. 1. Growth curves for toxigenic and non-toxigenic strains of *A. flavus* group
For full experimental details see the Materials and methods section. Toxigenic strains: NRRL-3240 (△) and NRRL-2999 (▲). Non-toxigenic strains: NRRL-3537 (○) and NRRL-5565 (●).

Fig. 2. GSH S-transferase activity profile: comparison of toxigenic and non-toxigenic strains of *A. flavus*
For full experimental details see the Materials and methods section. GSH S-transferase activity was estimated in the post-mitochondrial supernatant. Toxigenic strains: NRRL-3240 (△) and NRRL-2999 (▲). Non-toxigenic strains: NRRL-3537 (○) and NRRL-5565 (●). Values represented are the average of three determinations with variation less than 5%.
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Fig. 3. Effect of media on GSH S-transferase activity of A. flavus

Mycelia were grown as stationary cultures for 96 h in SLS (□) and GAN (■) media. For full experimental details see the Materials and methods section. The values are the average of two separate experiments done in triplicate. Toxigenic strains: NRRL-3240 and NRRL-2999; non-toxigenic strains: NRRL-3537 and NRRL-5565.

Table 1. Aflatoxin production by stationary culture of A. flavus on SLS and GAN

For full experimental details see the Materials and methods section. Values represented are the average of three determinations with variation less than 5%.

<table>
<thead>
<tr>
<th>Toxigenic strains</th>
<th>NRRL-3240</th>
<th>NRRL-2999</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium</td>
<td>Aflatoxins (μg/100 ml of medium)</td>
<td>Aflatoxins (μg/100 ml of medium)</td>
</tr>
<tr>
<td>B1 + B2</td>
<td>G1 + G2</td>
<td>Total</td>
</tr>
<tr>
<td>SLS</td>
<td>257.0</td>
<td>112.6</td>
</tr>
<tr>
<td>GAN</td>
<td>16.59</td>
<td>32.21</td>
</tr>
</tbody>
</table>

Table 2. Incorporation of [1-14C]acetate into aflatoxins by A. flavus NRRL-3240

The mycelia were grown at 28 ± 1 °C for 72 h as stationary cultures, 6 μCi of [1-14C]acetate (specific radioactivity 58.46 mCi/mmole) was added to each flask aseptically and incubated for 24 h. Aflatoxins were extracted from the medium with chloroform and resolved as described in the Materials and methods section. Isolated aflatoxins was counted in a liquid-scintillation spectrometer with counting efficiency of 92%. Values represented are averages of three determinations with variation less than 5%.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Aflatoxins...</th>
<th>B1 + B2</th>
<th>G1 + G2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLS</td>
<td>19428</td>
<td>8942</td>
<td>28371</td>
<td></td>
</tr>
<tr>
<td>GAN</td>
<td>4451</td>
<td>5865</td>
<td>10317</td>
<td></td>
</tr>
</tbody>
</table>

mycelia grown in SLS medium compared with those grown in GAN medium.

The kinetic parameters listed in Table 3 were deduced from Lineweaver-Burk plots. Although the apparent $K_m$ of the toxigenic strain was not significantly different from that of the non-toxigenic strain, the apparent $V_{\text{max}}$ of the toxigenic strain was significantly higher than that of the non-toxigenic strain.

Table 3. Kinetic parameters for GSH S-transferases of A. flavus

For full experimental details see the Materials and methods section. Values represented are average of three determinations

<table>
<thead>
<tr>
<th>Strain</th>
<th>Toxigenicity</th>
<th>$K_m$ (mM)</th>
<th>$V_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL-3537</td>
<td>Non-toxigenic</td>
<td>1.0</td>
<td>7.14</td>
</tr>
<tr>
<td>NRRL-3240</td>
<td>Toxigenic</td>
<td>0.8</td>
<td>14.28</td>
</tr>
<tr>
<td>NRRL-2999</td>
<td>Toxigenic</td>
<td>0.8</td>
<td>25.0</td>
</tr>
</tbody>
</table>

DISCUSSION

The ability of A. flavus to synthesize and metabolize aflatoxins has prompted the study of the cellular mechanisms of this fungi. The presence of cytochrome P-450-linked mono-oxygenases (Bhatnagar et al., 1982) and the epoxidation of AFB1 by microsomes prepared from A. flavus (M. Saxena, A. Allameh, H. G. Raj & K. G. Mukerji, unpublished results) shows the ability of the fungi to metabolize this carcinogen. Formation of AFB1-epoxide is the activation mechanism by which AFB1 elicits the carcinogenic response (Swenson et al., 1974). In a typical experiment the microsomes of the toxigenic strain of A. flavus catalysed binding of 20–30 pmol of AFB1/mg of exogenous DNA (calf thymus DNA). Upon addition of cytosol (hamster liver) the
AFB₁-DNA binding mediated by A. flavus microsomes was significantly reduced (50% of control), thereby substantiating the formation of the carcinogenic adduct with DNA as reported in mammalian tissues (Lotlikar et al., 1980, 1984).

The presence of GSH S-transferases in some fungi (Wackett & Gibson, 1982; Cohen et al., 1986) and in A. flavus (reported here) raises the possibility that this enzyme catalyses the conjugation of GSH to AFB₁-epoxide. In this paper we have examined the relation of cytosolic GSH S-transferases from A. flavus to aflatoxin synthesis. Factors influencing aflatoxin formation, such as growth period, medium etc., always enhanced GSH S-transferase activity in the toxigenic strain. This finding suggested a correlation between the GSH S-transferase activity and aflatoxin production. Since the non-toxigenic strain produces no aflatoxin, these factors have little effect on its GSH S-transferase activity.

Induction of GSH S-transferase activity by a variety of drugs has been shown in several mammalian species (Mukhtar & Bresnick 1976; David & Nerland, 1983; Awasthi et al., 1984; Sato et al., 1984; Singh et al., 1985). We have now shown the induction of this conjugating enzyme by endogenous aflatoxin in A. flavus (kinetic studies: Table 3). Similar results were obtained in rats with known inducers of GSH S-transferase, such as phenobarbital, butylated hydroxytoluene etc. (Kaplowitz et al., 1975), thereby indicating that substrate affinity for A. flavus GSH S-transferase was not altered by the action of inducers. The data presented here clearly show a positive correlation between aflatoxin formation and GSH S-transferase activity.

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REFERENCES
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