Inactivation of Aflatoxin $B_1$ by Using the Synergistic Effect of Hydrogen Peroxide and Gamma Radiation

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Aflatoxin $B_1$ is a highly toxic, mutagenic, carcinogenic, and teratogenic toxin produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus* (5, 7, 13). Aflatoxins are highly resistant to various physical and chemical treatments. Various chemicals have been used to destroy aflatoxins from contaminated foods. Sreenivasanmurthy et al. (14) have used hydrogen peroxide for effective detoxification of aflatoxin in peanuts. Exposure to UV radiation also reduces the toxicity of aflatoxin (1). Inactivation of aflatoxin with gamma radiation has also been studied (16). Aflatoxin is resistant to gamma radiation in dry conditions but becomes sensitive in aqueous solutions. Complete degradation of aflatoxin required a very high dose of gamma radiation (16).

In the present investigation, we report the synergistic effect of hydrogen peroxide and gamma radiation on the inactivation of aflatoxin. A systematic study was undertaken to standardize conditions for the maximum destruction of the toxin with minimum doses of gamma radiation. The degradation products were also checked for their biological activity by the Ames test.

**MATERIALS AND METHODS**

**Synthesis and purification of aflatoxin $B_1$.** Aflatoxin $B_1$ for routine experimental analysis was obtained by growing *Aspergillus parasiticus* NRRL 3240 in synthetic medium under static conditions as described earlier (12). After 7 days of incubation at 28 ± 2°C, aflatoxin was extracted and purified from the medium by the method of Steyn (15). Purity of aflatoxin was ascertained by high-pressure liquid chromatography (HPLC).

**Irradiation method.** Irradiation was carried out in a gamma chamber (model 900; provided by Bhabha Atomic Research Centre, Bombay, India). The radiation source was $^{60}$Co. Irradiation was performed at 28 ± 2°C at a dose rate of 6.6 krad/min. The desired concentration of aflatoxin was dissolved in 25 μl of dimethyl sulfoxide (DMSO) and irradiated in 1 ml of an aqueous system containing different concentrations of hydrogen peroxide. Controls contained only water. Irradiated aflatoxin was extracted with 2 ml of chloroform, and the organic phase was then evaporated to dryness. Residual aflatoxin was estimated by HPLC analysis. The degradation products were checked by thin-layer chromatographic (TLC) analysis, and their biological activity was also determined.

The effect of pH on the inactivation of aflatoxin was checked by taking 5% (wt/vol) H$_2$O$_2$ in an aqueous system with a different pH. The pH of the systems was adjusted by using NaOH and also by using 50 mM buffers of different pHs (sodium phosphate buffer, pH 7.0; Tris hydrochloride buffer, pH 8.0; and sodium glycine buffer, pH 9, 10, and 11). Controls contained only buffer. After irradiation, all the systems were acidified, and aflatoxin was extracted as described earlier.

**HPLC analysis.** HPLC was done with an LC 4A system (Shimadzu, Tokyo, Japan). Residual toxin was dissolved in 500 μl of methanol and estimated under the following conditions: column, Zorbax ODS (4.65 by 25 cm); mobile phase, methanol-water (60:40, vol/vol); flow rate, 1.0 ml/min; column temperature, 28°C. Detection of aflatoxin was done with a fluorescent detector (FD-DP 530; Shimadzu, Tokyo, Japan). Excitation and emission wavelengths were 362 and 425 nm, respectively. Aflatoxin $B_1$ was identified by confirming its retention time with standard aflatoxin $B_1$ obtained from Sigma Chemical Co.

**TLC analysis.** Irradiated and unirradiated aflatoxin was spotted on 0.25-mm-thick silica gel G plates (E. Merck, Bombay, India). The plates were developed with the mobile phase chloroform-acetone (9:1, vol/vol). Fluorescent spots were visualized with a UV transilluminator (Fotodyne Inc., New Berlin, Wis.).

**Biological assay.** Biological assay of control and treated aflatoxin was done by the Ames microosomal mutagenicity test with *Salmonella typhimurium* TA100 as the test strain and the plate incorporation assay method (11). Residual toxin was redissolved in 500 μl of methanol, and 10 μl of this was added per plate. The number of revertants obtained per plate was calculated, and the mean number of triplicates is given in the results.

**Detoxification of groundnuts.** Unshelled groundnuts (10 g) were artificially contaminated with approximately 10$^6$ spores of *Aspergillus parasiticus* NRRL 3240 and incubated for 12 days at 28 ± 2°C. Contaminated groundnuts were suspended in 25 ml of 5% hydrogen peroxide and irradiated at 200 krad. After the treatment, residual toxin was extracted by the method described by Coomes et al. (2) and estimated as described earlier.

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RESULTS

A 40% inactivation of aflatoxin was obtained when 50 μg of aflatoxin was irradiated at a dose of 1,000 krad (Fig. 1) in water. Addition of hydrogen peroxide to this system markedly increased the degradation of aflatoxin. Hydrogen peroxide at 1% and 3% yielded 37% and 43% inactivation of aflatoxin, respectively, at 200 krad. Increasing the dose to 1,000 krad did not significantly increase the inactivation of toxin, but 5% hydrogen peroxide caused 100% inactivation at a dose of 200 krad (Fig. 1).

When 50, 75, and 100 μg of aflatoxin was added to 1 ml of 5% hydrogen peroxide, it was observed that 100 krad was sufficient to inactivate 50 μg of toxin completely (Table 1). Aflatoxin at 75 and 100 μg showed 85 and 90% inactivation, respectively, at the same dose, whereas they were completely inactivated at 400 krad. Lower amounts of aflatoxin, i.e., 5 and 10 μg, required only 20 krad for complete inactivation in the presence of 5% hydrogen peroxide (data not presented).

Aflatoxin (50 μg) in a system containing distilled water (pH 5.2 to 5.4) and 5% H₂O₂ showed complete degradation upon irradiation with a dose of 100 krad. But only 60 and 40% degradation was achieved in systems having pHs of 7.0 and 11.0, respectively (data not presented).

Detection of degraded products. HPLC analysis revealed an additional peak which had a lower retention time (3.6 min) (Fig. 2b through d) than aflatoxin (5.9 min) (Fig. 2a). The control unirradiated samples gave only one peak of aflatoxin activity. The area of the new peak increased with increasing dose, with the aflatoxin peak showing a concomitant decrease. At 200 krad, no peak was detected, suggesting its complete degradation (Fig. 2e). When these samples were subjected to TLC analysis, similar results were obtained (Fig. 3). Irradiated samples showed a decrease in the intensity of the aflatoxin spot (Rf, 0.63), and new products were detected which showed lower Rf values (0.35) and were degraded at higher doses, suggesting complete degradation.

**Detoxification of groundnuts.** When artificially contaminated groundnuts were subjected to irradiation, the levels of aflatoxin decreased from 14.24 and 6.32 ppm (14.24 and 6.32 μg/g) to 2.82 and 1.67 ppm, respectively.

**Bioassay of aflatoxin.** Aflatoxin B₁, after irradiation at 400 krad, showed a complete loss of its toxicity in the Ames mutagenicity test (11). No revertants were obtained at 400 krad, whereas 208 revertants were obtained at 200 krad. The unirradiated toxin had a high (>10⁴) count of revertants.

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**TABLE 1.** Aflatoxin remaining after gamma irradiation in the presence of H₂O₂.

<table>
<thead>
<tr>
<th>Initial aflatoxin concn (μg)</th>
<th>Residual aflatoxin (μg) (% inactivation)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>50 krad</td>
</tr>
<tr>
<td>50</td>
<td>2.5 (95)</td>
</tr>
<tr>
<td>75</td>
<td>18.75 (75)</td>
</tr>
<tr>
<td>100</td>
<td>55 (40)</td>
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* H₂O₂ used was 1 ml of a 5% solution.
DISCUSSION

The aflatoxin contamination in different foodstuffs ranges from 200 to 750,000 μg/kg (9). Therefore, in the present study, we used high concentrations of aflatoxin. Earlier studies have shown that hydrogen peroxide could be used to detoxify contaminated foodstuffs, but for effective detoxification higher temperature and longer times were required without much change in the protein efficiency ratio (14). Studies on the use of gamma radiation for detoxification have revealed that very high doses are required to detoxify aflatoxin in aqueous solution, which is not within the permissible dose range for the irradiation of foodstuffs (16). Similar results were obtained in our study when aflatoxin was irradiated in aqueous solution. Only 40% of aflatoxin was irradiated in aqueous solution. Only 40% of aflatoxin was degraded with 1,000 krad (Fig. 1). In the present study, we have exploited the synergistic effect of hydrogen peroxide and gamma radiation to degrade higher concentrations of aflatoxin at lower doses. A 50 μg amount of aflatoxin can be degraded with 100 krad and 1 μl of 5% hydrogen peroxide. Under the same conditions, 100 μg of aflatoxin required a dose of 400 krad. But at any given dose of irradiation, the total amount of toxin degraded was the same irrespective of the initial toxin concentration (Table 1). When the concentration of hydrogen peroxide was lower, the degradation obtained was also lower. Hydrogen peroxide at 1 or 0.3% was not sufficient to completely degrade 50 μg of aflatoxin (Fig. 1). This suggests that hydrogen peroxide is a limiting factor in the system and that gamma radiation increases the effectiveness of degradation by hydrogen peroxide. At higher pHs, hydrogen peroxide is not very stable and is decomposed into oxygen and water. Therefore, the effective concentration of hydrogen peroxide was lower at a higher pH and degradation of aflatoxin was also lower (data not presented). Gamma radiation generates various free radicals which are highly active in damaging various biomolecules (8). Upon degradation, hydrogen peroxide also generates free radicals (4). It was also observed that hydrogen peroxide formed during the irradiation functions as a radiation sensitizer (6). In our system, hydrogen peroxide in association with gamma radiation produced a higher amount of free radicals in aqueous solution than the individual agents. This higher amount of free radicals generated may account for the lower doses of irradiation required for complete inactivation of aflatoxin in the presence of hydrogen peroxide.

The toxicity of treated aflatoxin also decreases, as seen by the Ames mutagenicity test. The degradation product did not show any mutagenicity in the Ames test. Similar observations were made by Vandyck et al. (16). Earlier studies also demonstrated the mutagenicity of the degraded products of aflatoxin after hydrogen peroxide treatment in a biological assay with ducklings and duck embryos (14).

The degradation products showed lower retention times and were more fluorescent (Fig. 2; the area of the peak of the degraded product was much higher than the aflatoxin peak). This observation was confirmed by TLC analysis. By visual observation of the TLC plates, it was observed that the intensity of the degraded products was higher than that of aflatoxin. TLC analysis also suggested that more than one fluorescent compound was formed upon the degradation of aflatoxins. After complete degradation, some fluorescence was still seen at the origin which was not present in the unirradiated sample. We have not identified the final product(s) formed during the degradation, but earlier studies showed that succinic acid is formed as one of the degradation products of aflatoxin when treated with hydrogen peroxide (3).

At present very few detoxification methods are available, of which ammonification has been extensively studied, but this method requires higher temperature and is time-consuming and expensive (10). Therefore, gamma radiation along with hydrogen peroxide would work as a much easier and less expensive method to detoxify contaminated foods, as it shows effective detoxification in artificially contaminated groundnuts and loss of mutagenicity in the Ames mutagenicity test. Animal toxicity studies are required before it can be commercially applied in detoxification of foodstuffs.

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LITERATURE CITED