A Research Note

Bacillus subtilis rec Assay for Quantification of Aflatoxins

MIGUEL D’AQUINO*, SILVIA BEJAR and ERNESTO BOLLINI

Department of Toxicology (Hygiene & Public Health), Faculty of Pharmacy and Biochemistry, Buenos Aires University, Junin 954, 1113 Buenos Aires, Argentina

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ABSTRACT

The Bacillus subtilis 1791 rec" assay was used to quantify genotoxic mycotoxins. This assay is based on detection of mycotoxin-produced DNA alterations arising from recombinational deficiency in rec" cells. Aflatoxin B1 showed a linear dose-response relationship when the inhibition halo was taken as a parameter for the evaluation procedure. Assays carried out with or without hepatic microsomal activation exhibited a similar response.

Mycotoxins are among the naturally occurring environmental contaminants of animal and human food, and have been reported as secondary fungal metabolites isolated mainly from cereal after storage at high temperature and humidity. Many studies have implicated mycotoxins as carcinogens (3,5,14,20). A strong correlation between carcinogenic and mutagenic activities has been reported for a large number of compounds (12), leading to development of low-cost short-term tests for rapid identification of potentially carcinogenic agents. One of these tests, the Rec Assay, is based on the inability of bacterial mutant strains (10,11,13) to repair DNA damage after cellular recombinations. The Bacillus subtilis rec" mutants are far more sensitive to DNA toxic compounds than the rec + wild type strain, a difference in sensitivity commonly known as the "rec effect."

Among the mycotoxins, aflatoxin is the most potent carcinogen (8,19). The biosynthesis, metabolism and detection of this widely distributed contaminant have been thoroughly investigated. Since this toxin is usually detected in edible cereal products, its presence represents a serious environmental and economic problem.

In this paper we report on the quantitative correlation between aflatoxin B1 toxicity and its mutagenic effect on B. subtilis rec to allow rapid screening of food products. A metabolically oxidized derivative of aflatoxin B1 has been reported as the active mutagenic compound (16). Since enzymatic oxidation is performed mainly in the liver, a hepatic microsomal metabolizing system was incorporated into the assay to evaluate the comparative action of the toxin and its metabolites (1,9).

MATERIALS AND METHODS

Bacterial strains

B. subtilis PB 1652 (Lys-3 Met B Trp C2) and PB 1791 (Met B Trp C2 Rec E) were kindly supplied by Dr. Giorgio Mazza, Genetic Institute of Pavia, Italy. To obtain better standardization and reproducible results, a standard technique was employed for bacterial spores.

Spores were prepared and purified according to Siccardi (15), then kept in distilled water at 4°C. The spore suspension used in the test contained 2 x 10^8 spores/ml.

Culture media

Britania Nutritive Agar was used as basal medium and top agar was prepared by dissolving Oxoid agar No. 3 (0.6%) in normal saline solution.

The components of Britania Nutritive Agar are: bacteriological peptone 5.0 g/L, beef extract 3.0 g/L, sodium chloride 8.0 g/L, and agar 15.0 g/L; pH 7.3.

Plate preparation

Ten ml of nutritive agar were poured into petri dishes and after solidification, plates were dried for 15 min at 60°C (bottom-agar). Test tubes containing 2 ml of top agar were maintained at 45°C and inoculated with 0.1 ml of spore suspension.

Whenever metabolic activation was required, 0.5 ml of S9 mix, prepared according to Ames (1), was added. The mixture was homogenized and immediately poured onto the plates. The petri dishes were kept at 4°C for 30 min for complete solidification, when absorbent 8-mm diameter paper discs were placed on the surface and the plate was inoculated with the toxin to be tested. For better diffusion, plates were kept at 4°C for 1 h and finally incubated at 37°C for 24 h.

Damage caused to bacterial DNA was calculated by measuring the diameter of the inhibition zone produced, or else by measuring the distance between the limits of the reservoir and the inhibition zone, following a technique similar to those used in antibiotic quantification (2,7).
BACILLUS SUBTILIS ASSAY FOR AFLATOXIN

Chemicals

Aflatoxin Bj, B2 and G2 were purchased from Sigma Chemical Co., and dimethylsulfoxide (DMSO) from Carlo Erba. A standard solution was prepared with 1 mg of aflatoxin dissolved in 1 ml of DMSO; the series of concentrations used in the test was prepared with the same solvent.

RESULTS AND DISCUSSION

Different mycotoxins produced various responses in the B. subtilis rec assay. This variation in sensitivity led to use of a high concentration to ensure a positive result. Since toxic action is determined by inhibition of bacterial growth, the effect was considered positive when the inhibition zone around the toxin showed a difference of more than 2 mm in radius between the rec- and rec+ strain (17).

Table 1 summarizes the reactivity in the B. subtilis rec assay according to toxin source and concentration, comparing our data with reports from other authors (16). On the basis of these results, the highly reactive aflatoxin B1 was chosen for the dose response study.

TABLE 1. Rec assay of mycotoxins.

<table>
<thead>
<tr>
<th>Fungi and mycotoxins</th>
<th>B. subtilis</th>
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<tbody>
<tr>
<td></td>
<td>Inhibition zone (mm)</td>
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<tr>
<td></td>
<td>μg/disc</td>
</tr>
<tr>
<td>Penicillium</td>
<td></td>
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<tr>
<td>Citrinin</td>
<td>20</td>
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<td>Patulin</td>
<td>20</td>
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<tr>
<td>Rugulosin</td>
<td>20</td>
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<td>Luteoskyrin</td>
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<td>Fusarium</td>
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<td>Zearalenone</td>
<td>100</td>
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<tr>
<td>Aspergillus</td>
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<tr>
<td>Aflatoxin B1</td>
<td>1</td>
</tr>
<tr>
<td>Aflatoxin B2</td>
<td>2</td>
</tr>
<tr>
<td>Aflatoxin B1</td>
<td>4</td>
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<tr>
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<td>6</td>
</tr>
<tr>
<td>Aflatoxin G1</td>
<td>100</td>
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</tbody>
</table>

*Difference in mm between the inhibition zones and reservoir limits with the B. subtilis Rec- and Rec+; +, >=2 mm; ++, >=5 mm.

Figure 1 shows a dose vs. inhibition zone plot for aflatoxin B1 with or without microsomal activation, and for aflatoxins B2 and G2, without microsomal activation. For aflatoxin B1 a linear relationship was observed between the log of the dose and the response expressed by the distance reservoir limit-area of inhibition in mm. However, neither aflatoxin B2 nor G2 exhibited activity in this assay.

Figure 1. Distance in mm between the limits of the reservoir and the area of inhibition versus log dose in μg tested per assay. — Aflatoxin B1 with metabolic activation. — — Aflatoxin B1 without metabolic activation. O---O Aflatoxin B2 without metabolic activation. □□□□ Aflatoxin G2 without metabolic activation.

mutagenic activity when other methods are not available, for instance when purification of a crude food extract is troublesome.

Use of a hepatic microsomal system for activation allows aflatoxins and structurally related metabolic products to be estimated, even in crude extracts. The assay performed with microsomal activation showed a slight, though non-significant increase in the response. The bacteria employed in the assay might possibly metabolize the aflatoxin efficiently to a genotoxic compound. Results indicate that the test may be carried out without hepatic microsomal activation even though its inclusion would lower the detection threshold. However, this last point deserves further research on the metabolic systems to improve the test’s reliability.

As the manifestation of genetic effects depends on diffusion of aflatoxin in the culture medium, the assay was modified to prevent growth before diffusion. Accordingly, a diffusion period of 1 or 24 h at 4°C for inoculated petri dishes with aflatoxin and microorganisms was done before incubation at 37°C. It was found that a 1-h diffusion period was sufficient and there was no significant improvement after 24 h of incubation at 4°C.

Additional tests were carried out with various Aspergillus culture extracts and contaminated peanut samples. A satisfactory response was achieved in the detection of aflatoxin B1 whenever this compound was present in appreciable concentrations. Since the maximum aflatoxin
level internationally accepted for cereals has been set at 15-20 µg/kg (ppb), this method allows its detection and quantification by processing 50 to 100-g samples. For the peanut assay, 50-g samples were extracted with 200 ml of chloroform. The extract was evaporated to dryness and the residue redissolved in 0.5 ml of DMSO. Determination was done by the described technique, but using 4 drops of the extract in a standard cylinder for antibiotic quantification instead of using paper discs. Results obtained with the sample were then compared with a DMSO dilution of standard aflatoxin B1 for its quantification. A 70-90 µg/kg aflatoxin concentration was detected by this method in products. Therefore, the improvement of working conditions during metabolic activation can optimize the response on food samples.

Although other genotoxicity assays may be applied to quantify aflatoxins, their implementation proves more complex than the rec- test. In the commonly used Ames test, lethal effects on the various strains employed are rather troublesome to monitor.

Even though this method is not so sensitive as other physico-chemical and immunologic procedures, it provides useful data on biologic activity.

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REFERENCES