A Microbiological Assay for Penicillic Acid

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ABSTRACT

Six bacterial cultures were studied in a search for an organism sensitive to penicillic acid suitable for use in a quantitative bioassay of this mycotoxin. A vegetative culture and a commercially prepared spore suspension of Bacillus subtilis were both sensitive to as little as 1 µg/mL of penicillic acid and exhibited a linear relationship between 1 and 100 µg/mL. The bioassay method was comparable in accuracy to thin layer chromatographic assay. The procedure was used to verify the biological activity of sample extracts, as well as to quantitate penicillic acid concentration in samples of liquid media and corn. The bioassay is sensitive, rapid (15-17 h), simple and inexpensive.

Use of microbiological assay techniques to confirm the presence of mycotoxins and supplement results of thin layer chromatography (TLC) detection has been employed by several workers. Burmeister and Hesseltine (4) reported that of 392 different microorganisms surveyed for sensitivity to aflatoxins, Bacillus megaterium NRRL B-1368 was most sensitive. Clements (5) later used this organism in developing a rapid confirmatory test and quantitative bioassay for aflatoxin B1. B. megaterium NRRL B-1368 has also been reported to be sensitive to ochratoxin A (6) and a confirmatory test for ochratoxin A using this organism has been reported (16). Broce et al. (3) reported a quantitative bioassay and confirmatory test ochratoxins A and B, using Bacillus cereus var. mycoides (LSU) (Louisiana State University). Stott and Bullerman (17) reported that B. megaterium NRRL B-1368 was also sensitive to patulin and was a suitable test organism for an accurate quantitative bioassay of the toxin. Reiss (14) also developed a sensitive bioassay for patulin using a spore suspension of B. subtilis.

Penicillic acid, first isolated in 1913 by Alsberg and Black (2), is produced by numerous mold species commonly found in foods and feeds. In previous work, gram-positive and gram-negative bacteria have been found to be sensitive to penicillic acid (7). In those studies, the activity of the compound was reported in terms of the highest dilution required to prevent growth using the streak plate method with Escherichia coli, Staphylococcus aureus and Bacillus subtilis (7). The cylinder plate method was used with S. aureus and E. coli (8). Tube dilution methods have also been used to assay penicillic acid with S. aureus, E. coli and Salmonella typhi (11,12). This method has also been used quantitatively, with B. subtilis (8 µg/mL), E. coli (64 µg/mL) and S. aureus (16 µg/mL) by Kavanagh (9). With these methods either quantitative results were not always reported, or sensitivity was poor.

The objective of this study was to develop a more sensitive quantitative bioassay for penicillic acid that could be used as a sensitive, rapid, simple and inexpensive means of detecting biological activity in sample extracts.

MATERIALS AND METHODS

Cultures

Cultures of E. coli, S. aureus and B. subtilis were obtained from the Department of Food Science and Technology culture collection. B. megaterium NRRL B-1368 and B. cereus var. mycoides (LSU) were obtained previously for bioassay work with patulin and were maintained in our laboratory. A commercially prepared B. subtilis spore suspension (Difco) was also used. These organisms were selected to test for sensitivity to penicillic acid because they had either been reported in the literature to be sensitive to penicillic acid or had been reported to be sensitive to patulin which is believed to have a mode of toxicity similar to that of penicillic acid. Cultures were maintained on nutrient agar and stored at refrigerated temperatures.

Mycotoxin standard

Crystalline penicillic acid was diluted with reagent grade chloroform to give a concentration of 1 µg/dl. The purity and concentration of the solution were confirmed using ultraviolet absorption spectrophotometry and thin layer chromatography (10,15).

Media and inoculum preparation

Tryptone-yeast extract-glucose (TYG) broth and agar (7) were used for this assay. The cultures were prepared by inoculating a tube of TYG broth with the appropriate microorganism and incubating at 35-37 C for 15-20 h until approximately 70% transmittance was obtained as...
measured at 520 nm in 1-cm cell (Bausch and Lomb Spectronic 20 spectrophotometer). Transmittance was adjusted with sterile water when necessary. Two ml of the inoculum preparation was added to each 100 ml of melted and tempered (50 C) TYG agar when assaying with S. aureus, E. coli, B. megaterium, and B. cereus var. mycoides; 1 ml/100 ml TYG agar was used for B. subtilis and the B. subtilis spore suspension. After the inoculum had been added, the TYG agar was swirled to mix the solution to insure uniform distribution, and 10 ml of the seeded agar was aseptically pipetted into sterile glass petri dishes (15 x 100 mm) and allowed to solidify.

Assay procedure

Blank antibiotic discs (6.35 mm in diameter, No. 740, Schleicher and Schuell, Keene, NH) were placed upon a wire mesh support to insure that the disc absorbed all the solvent that was delivered to it. To determine sensitivity limits and linear response of each microorganism studied, discs were prepared containing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50 and 100 µg concentrations of penicillic acid standard solution. Discs containing 5 and 20 µl of solvent control were also prepared. Solvent control and standard toxin solutions were slowly applied to discs dropwise using a microliter syringe. The discs were allowed to dry approximately 10 min and then four discs (impregnated side down) were evenly spaced on the agar surface of each prepared plate. The plates were then inverted and pre-incubated at 5 C for 30 min to allow uniform diffusion into the agar. After preincubation, plates were incubated overnight (15-17 h) at 35-37 C. The inhibition zones were then measured with vernier calipers to the nearest 0.1 mm; the diameter of the paper disc (6.35 mm) was subtracted to obtain the corrected zone of inhibition on the x-axis of logarithmic graph paper, standard curves for penicillic acid inhibition of the various cultures were obtained. The standard curve maintained its linearity. The results obtained using vegetative cultures of B. subtilis were similar, indicating that either spores or vegetative cultures could be used.

The quantitative bioassay separately using vegetative cultures and spores of B. subtilis was compared to quantitative measurement of penicillic acid by TLC using visual estimation of concentration after derivatization with phenylhydrazine (15). Twelve chloroform extracts of several liquid media cultures of a penicillic acid producing Penicillium sp. were quantitated by the two methods. For the bioassay 1-, 5-, 10- and 20-µl volumes were also spotted. The value obtained by TLC quantitation was the average of three independent observations for each sample. The results were compared statistically by the analysis of variance method. There

RESULTS AND DISCUSSION

Table 1 gives the lowest concentration of penicillic acid that caused a measurable zone of inhibition with each of the microorganisms tested. B. megaterium NRRL 1368 and B. cereus var. mycoides LSU were the least sensitive to penicillic acid, and S. aureus and E. coli were equally sensitive to intermediate amounts of the mycotoxin. Vegetative cultures and the commercial spore suspension of B. subtilis were the most sensitive to penicillic acid, with inhibition occurring with as little as 1 µg of the compound. The chloroform control discs gave no inhibition.

When the penicillic acid concentration (µg/disc) was plotted on the y-axis and the corresponding average corrected zone of inhibition on the x-axis of semilogarithmic graph paper, standard curves for penicillic acid inhibition of the various cultures were obtained. The standard curve obtained with B. subtilis spores is shown in Fig. 1.

Regression analysis of the two variables for the curve gave a correlation coefficient of 0.986, which is indicative of a strongly positive linear relationship between the

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Penicillic Acid (µg)</th>
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<tbody>
<tr>
<td>S. aureus</td>
<td>3.5</td>
</tr>
<tr>
<td>E. coli</td>
<td>3.5</td>
</tr>
<tr>
<td>B. megaterium NRRL B-1368</td>
<td>8-12</td>
</tr>
<tr>
<td>B. cereus var. mycoides LSU</td>
<td>12</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>1</td>
</tr>
<tr>
<td>B. subtilis (spore suspension)</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 1. Standard curve for bioassay of penicillic acid using Bacillus subtilis spores.
was no significant difference ($P = 0.01$) between the chemical and biological quantitation methods. The bioassay was also compared with TLC using an extract obtained from corn that had been inoculated with a known penicillic acid producing *Penicillium* sp. The corn was extracted by the method of Pohland and Allen (13). The bioassay and TLC quantitations showed no significant differences. Again, there was very little difference in sensitivity to penicillic acid between vegetative cells and spores of *B. subtilis*.

The work reported here clearly indicates the suitability of *B. subtilis* for a quantitative bioassay of penicillic acid. A definite linear relationship existed between the concentration of penicillic acid and the zone of inhibition of *B. subtilis* when using the disc plate technique of microbiological assay. This method was more sensitive than those previously reported (7,9) and is comparable to the sensitivity reported for microbiological assays of other mycotoxins.

The accuracy and quantitative capability of this bioassay method compare favorably with quantitative thin layer chromatography. The technique, using commercial *B. subtilis* spore suspensions, offers a simple, rapid, inexpensive confirmatory and quantitative method that can be used to supplement TLC in determining the presence and concentration of penicillic acid in sample extracts.

REFERENCES


