Sodium Benzoate in the Control of Growth and Aflatoxin Production by Aspergillus parasiticus

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(Received for publication September 15, 1986)

ABSTRACT

Sodium benzoate, 0.0, 0.1, 0.2, 0.3 or 0.4%, was added to a glucose-yeast-salts medium which was inoculated with 1 ml of a spore suspension containing 10^8 conidia of Aspergillus parasiticus NRRL 2999 and then was incubated at 28°C. Cultures were analyzed after 3, 7 and 10 d for mycelial dry weight, pH and accumulation of aflatoxin B_1 and G_1. Amounts of aflatoxin produced were determined using reversed-phase high performance liquid chromatography (HPLC). The percentage of inhibition or stimulation by the additive was used to make comparisons between treatments and control. Generally, increasing the concentration of sodium benzoate increased the percentage of inhibition at the end of incubation (10 d). However, the average accumulation of mycelial dry weight was greater in the presence of benzoate than in its absence, with the greatest increase occurring when the medium contained 0.3% sodium benzoate.

Although aflatoxin has been extensively investigated (12, 13), little information has accumulated on the effect of sodium benzoate on growth and aflatoxin production by aspergilli. Hence, the present study was undertaken to determine and characterize how various concentrations of sodium benzoate affect growth and aflatoxin production by A. parasiticus NRRL 2999 in a glucose-yeast-salts medium at 28°C.

MATERIALS AND METHODS

Medium

A glucose-yeast-salts medium (14) was used throughout this experiment. The medium contained the following per liter: glucose, 60 g; (NH_4)_2SO_4, 4 g; KH_2PO_4, 10 g; MgSO_4·7H_2O, 2 g; Na_2B_4O_7·10H_2O, 0.7 mg; (NH_4)_6Mo_7O_24·4H_2O, 0.5 mg; Fe_2(SO_4)_3·6H_2O, 10 mg; CuSO_4·5H_2O, 0.3 mg; MnSO_4·H_2O, 0.11 mg; ZnSO_4·7H_2O, 17.6 mg; and 20 g of yeast extract.

Mold

A. parasiticus NRRL 2999 was obtained from the Northern Regional Research Center, U.S.D.A., Peoria, IL. The mold was grown on Mycological agar slants that were incubated at 28°C. After 7 d, spores were harvested using sterile distilled water with glass beads; the glass beads helped to dislodge spores from the mycelium when shaken. The spore suspension was pooled in a sterile 125-ml Erlenmeyer flask. The number of spores present per milliliter was determined by plate count.

Three hundred milliliters of the medium was dispensed into each of a series of 500-ml Erlenmeyer flasks and 0.0, 0.1, 0.2, 0.3 and 0.4% sodium benzoate added. The initial pH of the contents of each flask was determined using a pH meter, (model 601A/digital, Ionanalyzer, Orion Research, Cambridge, MA). The pH was adjusted to 5.5 using 0.1 N KOH. Twenty-five milliliters of the medium was dispensed into each of a series of 125-ml Erlenmeyer flasks. The medium was autoclaved at 121°C for 15 min, cooled, and inoculated with 1 ml of the appropriate spore suspension. Inoculated flasks were incubated at 28°C and the contents analyzed after 3, 7 and 10 d of incubation.
Analysis
Contents of flasks, with and without (which served as the control) sodium benzoate, were analyzed in triplicate for pH, dry weight of mycelium and aflatoxin.

Dry weight of mycelium. The medium was filtered through a modified Büchner funnel (14) fitted with preweighed Whatman No. 1 filter paper (12.5 cm in diameter) and attached to a 125-ml separatory funnel. Vacuum was applied to the funnel using an aspirator. The mycelium was washed with 10 ml of distilled water followed by 10 ml of chloroform (AR, Mallinkrodt). The water was transferred to the modified Büchner funnel and the chloroform was transferred directly into the separatory funnel. We observed that when the chloroform wash was added to the Büchner funnel, it could not pass through because an emulsion was formed. The filter paper with the mycelium was dried at 50°C for 48 h, cooled in a desiccator, and weighed.

Extraction of aflatoxin. Extraction of aflatoxin was done as described by Yousef et al. (15), but with some modifications. As soon as the filter paper with mycelium were removed from the Büchner funnel, 40 ml of chloroform was added to the separatory funnel and the contents were shaken for 2 min. The liquid phases were allowed to separate and the chloroform layer was passed through 10 g of anhydrous granular Na$_2$SO$_4$ in a funnel and collected in a 250-ml round-bottom flask. Extraction was repeated once more with 50 ml of chloroform and the chloroform phase was combined with the earlier phase in the round-bottom flask. Chloroform in the round-bottom flask was dried using a rotary evaporator (Rotavapor, Bühì, Brinkmann, Westbury, NY); the temperature of the water bath was between 50 and 55°C. The resulting dried films were redissolved in known volumes of methanol (LC grade, Baker, Phillipsburg, NJ).

Analysis of aflatoxin. Aflatoxins B$_1$ and G$_1$ were determined using a reverse phase HPLC system. The column was reverse-phase 5 μC$_18$ (Nova Pak No. 86344, Waters, Milford, MA), the detector was of the fluorescence type (Waters, Model No. 420) with an aflatoxin lamp (Waters, No. 78409), 365-nm excitation filter (Waters, No. 78225) and 425-nm emission filter (Waters, No. 78155); and the injector (Model 7120, Rheodyne) was equipped with a 10 μl sampling loop. The pump was Waters, Model 7120 and the recorder was Model 252A, Linear.

Aflatoxins B$_1$ and G$_1$ standards were obtained from Sigma (St. Louis, MO), and dissolved in a known volume of methanol to give the desired concentrations. The mobile phase used was 1% acetic acid and acetonitrile (LC grade, Baker), 65:35, as described by Waters (2). The mobile phase before use was degassed for 3 min using vacuum and a sonicator.

Aflatoxins were assayed by injecting 50 μl of the methanol extract into the HPLC system and concentrations were determined by comparing peak heights to those of the standard. Before analysis of samples, aflatoxin B$_1$ and G$_1$ standards were injected until uniform peak heights were obtained. Each sample was injected twice.

Analysis of data
(a) Arithmetic average rate of accumulation of aflatoxin during intervals (μg/d) =

\[
\frac{\text{aflatoxin attained at the end of intervals (μg/25 ml)}}{\text{number of days in interval}}
\]

(b) Arithmetic average rate of degradation of aflatoxin during intervals (μg/d) =

\[
\frac{\text{amount of toxin degraded (μg/25 ml)}}{\text{number of days in interval}}
\]

(c) Average rate of net toxin production during the period of incubation (μg/d) =

\[
\frac{\text{maximum accumulated toxin – amount of toxin degraded}}{\text{overall period of incubation}}
\]

(d) Arithmetic average rate of increase of dry weight of mycelium (g/d) =

\[
\frac{\text{dry weight of mycelium attained at the end of intervals (g)}}{\text{number of days in interval}}
\]

(e) Arithmetic average rate of decrease in dry weight of mycelium (g/d) =

\[
\frac{\text{decrease in dry weight of mycelium (g)}}{\text{number of days in interval}}
\]

(f) Average rate of net dry weight of mycelium during the period of incubation (g/d) =

\[
\frac{\text{maximum dry weight of mycelium – amount of autolysis}}{\text{overall period of incubation (10 d)}}
\]

(g) Average rate of net toxin produced (μg) by 1 g of dry mycelium weight in one day =

\[
\frac{\text{average rate of net toxin produced during the period of incubation (μg/d)}}{\text{average rate of net dry mycelium weight during the period of incubation (g/d)}}
\]

(h) Percentage of inhibitory or stimulatory effect of food additive =

\[
\frac{B - A \times 100}{A}
\]

where A = Average rate of net toxin produced by 1 g of dry mycelium in 1 d by the mold in a medium containing no additive.

where B = Average rate of net toxin produced by 1 g of dry mycelium in 1 d by the mold in a medium containing the additive, 0.2%, for example.

RESULTS AND DISCUSSION

pH of medium

Results in Table 1 indicate that the pH of the medium decreased after 3 d; the initial pH of the medium was adjusted to 5.5. This initial decrease in pH was influenced by the concentration of sodium benzoate and the amount of mold growth that occurred. The rate of pH change was slower in the medium containing 0.2, 0.3 or 0.4% sodium benzoate than in the medium with 0.0 or 0.1% sodium benzoate. However, the pH after 3 d

JOURNAL OF FOOD PROTECTION, VOL. 50, APRIL 1987
TABLE 1. Mycelial dry weight, pH and aflatoxin production by A. parasiticus in sodium benzoate containing cultures.

<table>
<thead>
<tr>
<th>Sodium benzoate concentration (g/100 ml)</th>
<th>Period of incubation (days)</th>
<th>Mycelial dry weight (g/25 ml of culture)</th>
<th>pH</th>
<th>Aflatoxin produced (µg/25 ml of culture)</th>
<th>B1</th>
<th>G1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>0.430</td>
<td>3.30</td>
<td>1490.85</td>
<td>9160.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.575</td>
<td>6.32</td>
<td>6100.00</td>
<td>46550.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.428</td>
<td>7.07</td>
<td>2050.00</td>
<td>21320.00</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>3</td>
<td>0.170</td>
<td>5.03</td>
<td>661.50</td>
<td>2240.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.663</td>
<td>6.14</td>
<td>4450.00</td>
<td>25602.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.456</td>
<td>7.20</td>
<td>2485.50</td>
<td>21500.00</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>3</td>
<td>0.098</td>
<td>5.33</td>
<td>112.50</td>
<td>280.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.687</td>
<td>6.21</td>
<td>3450.00</td>
<td>18462.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.419</td>
<td>7.00</td>
<td>2183.25</td>
<td>16452.00</td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>3</td>
<td>(0.00039)</td>
<td>5.44</td>
<td>no toxin</td>
<td>no toxin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.679</td>
<td>3.38</td>
<td>3850.00</td>
<td>21708.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.754</td>
<td>5.54</td>
<td>2439.5</td>
<td>28600.00</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>3</td>
<td>no growth</td>
<td>5.25</td>
<td>no toxin</td>
<td>no toxin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.0849</td>
<td>5.49</td>
<td>350.02</td>
<td>1017.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.54</td>
<td>6.64</td>
<td>1768.0</td>
<td>13526.00</td>
<td></td>
</tr>
</tbody>
</table>

always was in the range where antimicrobial activity of benzoic acid can be expected.

After 7 d of incubation, the pH increased in the medium containing 0.0, 0.1, 0.2 and 0.4% sodium benzoate and decreased in the medium with 0.3% of sodium benzoate. This may have resulted because the amount of growth that occurred from 3 to 7 d in the medium containing 0.3% of sodium benzoate was greater than in the medium with other amounts of the chemical. After 10 d of incubation, generally, the pH had increased; the rate of increase was influenced by the amount of mold growth that occurred from 7 to 10 d. This was greater in the medium with 0.3 and 0.4% sodium benzoate than with 0.0, 0.1 and 0.2% of the chemical. The rate of change in pH appears related to the change in amount of growth that occurred during the 7 to 10-d incubation interval. When the mold was still growing, the rate of increase in pH during this period was greater than that observed when autolysis of the mycelium began at the end of the growth phase (14). These results agree with those reported by several investigators including Buchanan (4), who studied the effect of sodium acetate on growth and aflatoxin production by A. parasiticus NRRL 2999, El-Gendy and Marth (9), who studied growth and aflatoxin production by A. parasiticus in the presence of Lactobacillus casei, and Yousef and Marth (14) who measured growth and synthesis of aflatoxin by A. parasiticus in the presence of sorbic acid.

**Dry weight of mycelium**

Results in Tables 1 and 2 indicate that sodium benzoate affected growth of the mold. The average amount of mycelial dry weight decreased during the period of incubation from 0 to 3 d in cultures containing sodium benzoate as compared to cultures free of sodium benzoate; 0.4% of the chemical completely inhibited mold growth during the 0 to 3-d interval. The medium free of sodium benzoate permitted more growth than did the medium with 0.1, 0.2 or 0.3% of the chemical during the period of incubation from 0 to 3 d. Such an effect can be attributed to an increase in the lag phase of the mold caused by the presence of sodium benzoate in the medium (8).
Effect of sodium benzoate on the average rate of accumulation and degradation of aflatoxin B₁ and G₁ (gld) produced by A. parasiticus incubated in 25 ml of medium at 28°C for 10 d.

<table>
<thead>
<tr>
<th>Period of incubation</th>
<th>Amount of sodium benzoate (%) in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B₁</td>
</tr>
<tr>
<td>0-3 d</td>
<td>496.95</td>
</tr>
<tr>
<td>3-7 d</td>
<td>1152.29</td>
</tr>
<tr>
<td>7-10 d</td>
<td>1350.0a</td>
</tr>
</tbody>
</table>

Average rate of net toxin production during the period of incubation (10 d) 205.0 | 2132.0 | 248.6 | 2150.0 | 218.33 | 1645.2 | 244.0 | 2860.0 | 176.8 | 1352.6

aAverage rate of toxin degradation.
bNo toxin produced.

Effect of sodium benzoate on the average rate of net aflatoxin B₁ and G₁ produced by 1 g of mycelium (dry weight) in 1 d by A. parasiticus incubated at 28°C for 10 d.

<table>
<thead>
<tr>
<th>Type of aflatoxin</th>
<th>Percent of sodium benzoate in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Aflatoxin B₁, Net amount inhibition or stimulation (%)</td>
<td>4767.4</td>
</tr>
<tr>
<td></td>
<td>4.29a</td>
</tr>
<tr>
<td>Aflatoxin G₁, Net amount inhibition or stimulation (%)</td>
<td>49581.4</td>
</tr>
<tr>
<td></td>
<td>13.27b</td>
</tr>
<tr>
<td>Aflatoxin B₁ + G₁, Net amount inhibition or stimulation (%)</td>
<td>54348.8</td>
</tr>
<tr>
<td></td>
<td>11.73b</td>
</tr>
</tbody>
</table>

aStimulation.
bInhibition.

Maximum mycelial growth occurred after 7 d in the medium containing 0.0, 0.1 and 0.2% sodium benzoate, whereas in the presence of 0.3 and 0.4% the mold was still growing during the 7 to 10-d interval of incubation (Table 1). Results in Tables 1 and 2 further indicate that a decrease in mycelial weight occurred during the interval from 7 to 10 d in the absence of or in the presence of 0.1 and 0.2% sodium benzoate, whereas the weight of the mycelium was still increasing in the presence of 0.3 and 0.4% of the compound. Such a decrease in the dry weight of mycelium during the final stages of the growth cycle can be explained by bioautolysis, which may have occurred at that time and resulted in loss of soluble intracellular solutes which were released during hydrolysis of the mycelium during the filtration step of the analysis (14).

Overall, at the end of incubation the average net dry mycelium weight produced during the period of incubation (10 d) was greater in the medium with 0.1, 0.3 and 0.4% rather than in the medium with 0 or 0.2% sodium benzoate. Similar results were reported by Yousef and Marth (14) when they studied growth and synthesis of aflatoxin by A. parasiticus in the presence of sorbic acid.

Production of toxin
The work reported in this paper was done, in part, to find a more precise measure to demonstrate the stimulatory or inhibitory effect of food additives on growth and aflatoxin biosynthesis and accumulation by A. parasiticus. The amount of toxin produced by a unit amount of organism in a unit of time is a constant characteristic of the strain and environmental conditions. Furthermore, this measure (the percentage of inhibitory or stimulatory effect of a food additive) attempts to compare production of aflatoxin under different environmental conditions. The measure tested the average rate of net toxin produced by 1 g of dry mycelium in 1 d during the period of incubation and relative comparisons were made between the control and treatments (Tables 2, 3 and 4).

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Results in Tables 1-4 indicate that biosynthesis and accumulation of aflatoxin were influenced most by concentration of sodium benzoate in the medium, length of the incubation period and mycelial growth; others (4,8,14) made similar observations in their studies on effect of certain salts on aflatoxin production by A. parasiticus. Data in Table 3 indicate that for the 0 to 3-d incubation interval the average rate of aflatoxin production was greater in the control than in the medium containing 0.1 or 0.2% sodium benzoate. No toxin was produced when the medium contained 0.3 or 0.4% of the chemical. This is true for aflatoxin B₁ and G₁.

The amount of aflatoxin G₁ produced by A. parasiticus in the absence or presence of sodium benzoate was greater than that of B₁, this was true after 3, 7 or 10 d at 28°C (Table 1). These results agree with those obtained by Bullerman et al. (5) when they studied aflatoxin production in aged dry salamis and aged country cured hams.

It is important to consider the average rate of net toxin produced during the period of incubation (10 d). In general, increasing the concentration of sodium benzoate decreased the total amount of aflatoxin produced during the period of incubation (Table 4). From the same table we can see that sodium benzoate at concentrations of 0.1 or 0.2% stimulated toxin production (B₁). Presumably, a low sodium benzoate content within the cytoplasm is favorable for efficient operation of various enzyme systems (3) involved in toxin production, particularly of B₁.

Generally, the percentage of inhibition by sodium benzoate or production of aflatoxin B₁ plus G₁ during the entire period of incubation (10 d) (Table 4) was inversely proportional to the concentration of sodium benzoate. This is true also for G₁ (Table 4). Cruces and Richert (7) reported that at pH 2.3 to 2.4 only 0.02-0.03% of sodium benzoate was required to prevent growth of most fermentation organisms tested. From the foregoing it seems that this measure was useful to make relative comparisons to demonstrate the effect of an additive on aflatoxin production. Achmoody and Chipley (1) imply that aflatoxin production per unit of growth is a more precise measure of the mold’s ability to produce aflatoxin than are the absolute values for aflatoxin accumulation.

Results of our study indicate that 0.4% sodium benzoate prevented growth and toxin production at the beginning of the incubation period (0-3 d), whereas this was not true for the incubation intervals from 3 to 7 d and from 7 to 10 d. Overall, the percentage of inhibition by sodium benzoate increased as concentration of the chemical increased. However, sodium benzoate at concentrations used in these experiments did not prevent growth and aflatoxin production by A. parasiticus incubated at 28°C for 10 d.

ACKNOWLEDGMENTS

Research supported by the College of Agricultural and Life Science, University of Wisconsin-Madison, and the American-Mideast Educational and Training Services through a Peace Fellowship to Fathy E. El-Gazzar. Special thanks for support of F.E.E. also go to the Egyptian Government.

REFERENCES