The Influence of Divalent Cations and Chelators on Aflatoxin B₁ Degradation by Flavobacterium aurantiacum

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ABSTRACT

The influence of divalent cations (Mg²⁺ and Ca²⁺) and chelators (EDTA and 1,10-phenanthroline) on aflatoxin B₁ (AFB₁) degradation by Flavobacterium aurantiacum was determined in an effort to elucidate the possible manner by which this organism degrades AFB₁. AFB₁ (10 μg/ml) was added to 72-h cultures of F. aurantiacum that had been washed and resuspended in phosphate buffer (pH 7.0). High-performance liquid chromatography was used to determine AFB₁ concentration in these cultures. Incubating cells with 0.1, 1, and 10 mM Ca²⁺ for 48 h significantly increased AFB₁ degradation by 11.8, 13.5, and 14.0%, respectively, compared with F. aurantiacum cells alone. Likewise, incubation with 0.1, 1, and 10 mM Mg²⁺ for 48 h significantly increased AFB₁ degradation by 13.8, 13.3, and 13.1%, respectively. Incubating the bacterium with either divalent cation for 16 and 24 h did not significantly affect AFB₁ degradation (P ≤ 0.05). Addition of 0.1, 1, and 10 mM EDTA and 0.1 and 1 mM 1,10-phenanthroline resulted in significant increases in AFB₁ degradation after 24 h. Significantly less AFB₁ degradation was observed using 10 mM 1,10-phenanthroline after 24-h incubation. These results suggest the involvement of Mg²⁺ and Ca²⁺ cations in AFB₁ degradation by F. aurantiacum.

Aflatoxin B₁ (AFB₁) is considered the most potent carcinogen of all the naturally produced toxins (8). Many crops, including peanuts, walnuts, corn, cottonseed, almonds, and pecans, are often naturally contaminated with aflatoxins during production, harvest, storage, and transportation (4). Research has been undertaken toward attaining successful and improved methods of aflatoxin removal and/or detoxification. The common methods of decontamination include physical segregation, which is inexpensive but labor intensive (1), and inactivation, which can be done by physical, chemical, or biological methods (13). Physical and chemical methods, although effective, are expensive and result in losses in nutritional and organoleptic qualities of the food product or treated feed (17). Microorganisms that are capable of degrading aflatoxin produce aflatoxins themselves (13) and have not undergone sufficient research for their use in practical situations. Ciegler et al. (2) screened more than 1,000 microorganisms for aflatoxin-degrading ability. They found only a single bacterium, Flavobacterium aurantiacum NRRL B-184, that could remove AFB₁ from solution. This bacterium can remove aflatoxin from a variety of food commodities, including milk, corn oil, peanut butter, corn, soybeans, peanuts, and peanut milk (2, 8). The potential value of this organism in biological decontamination procedures of these and other foods and feeds has been suggested by these authors.

Earlier work by Line and Brackett (10) showed that higher cell populations (approximately 1 × 10¹⁰ CFU/ml) and older (72-h) cultures of F. aurantiacum were more effective in removing AFB₁ from solution than younger (24- or 48-h) cultures. Heat-inactivated (nonviable) cells were unable to facilitate toxin removal. This organism also degrades aflatoxin to water-soluble and chloroform-soluble products and gaseous carbon dioxide (12). Line and Brackett (11) postulated a mineralization phenomenon of degradation, since additional energy sources did not enhance the removal of AFB₁.

The purposes of the research described herein were to (i) identify the role of the divalent cations (Ca²⁺ and Mg²⁺) in AFB₁ degradation by F. aurantiacum, (ii) establish the effect of chelators (EDTA and 1,10-phenanthroline [OPT]) on AFB₁ degradation, and (iii) determine the effect of the divalent cations (Ca²⁺ and Mg²⁺) in the presence of chelators. Magnesium and calcium were chosen for study because they are natural activators of a wide range of enzymes. The chelators used in this study included EDTA and OPT. OPT is a chelator of a wide range of metal ions with a high affinity for divalent Cu, Fe, Zn, and trivalent Fe.

MATERIALS AND METHODS

Cell culture. F. aurantiacum NRRL B-184 was obtained from Dr. L. K. Nakamura (U.S. Department of Agriculture, Northern Regional Research Center, Peoria, Ill.). The cells were grown in tryptic soy broth (Difco Laboratories, Inc., Detroit, Mich.) at 30°C and maintained by transferring at approximately 3-day intervals to fresh broth and tryptic soy agar slants in a similar manner as that described by Line and Brackett (10).

Determination of AFB₁ degradation. The ability of F. aurantiacum cells to degrade AFB₁ from solution over time was examined in the presence or absence of divalent cations (Ca²⁺, Mg²⁺), the presence or absence of chelators (EDTA, OPT), or the...
FIGURE 1. AFB1 degradation by F. aurantiacum at 30°C in the presence of divalent Ca or divalent Mg. Error bars represent standard deviations. Control refers to AFB1 degradation by F. aurantiacum in the presence of AFB1 alone.

The presence of divalent cations and chelators added together (Sigma Chemical Co., St. Louis, Mo.). The divalent cations were obtained in their chloride forms. AFB1 (Sigma) was obtained in its crystalline form and resuspended in chloroform as described previously (10). F. aurantiacum cells were grown in 500 ml of tryptic soy broth at 30°C for 72 h with constant agitation (150 rpm) in a similar manner to one described earlier (6). Portions of the cell solution were serially diluted in phosphate buffer (0.1 M phosphate buffer, pH 7.0) and enumerated by surface spreading on tryptic soy agar. Equal portions (10 ml) of the cell solution were added to 15-ml test tubes, pelleted by centrifugation at 1,800 g for 10 min, washed, and resuspended in 9 ml of phosphate buffer. AFB1 (10 μg/ml); AFB1 (10 μg/ml) and chelator (0.1, 1, or 10 mM); AFB1 (10 μg/ml) and divalent cation (0.1, 1, or 10 mM); or AFB1 (10 μg/ml), divalent cation (0.1, 1, or 10 mM), and chelator (1 mM) were added to these samples. Controls used included (i) AFB1, (ii) AFB1 and chelator, (iii) AFB1 and divalent cation, and (iv) AFB1, divalent cation, and chelator in phosphate buffer without the addition of cells. Samples in phosphate buffer with effectors only (without added AFB1) were also used as controls. Following incubation at 30°C with agitation at 150 rpm at various time intervals of 0, 16, 24, and 48 h, cells were again pelleted by centrifugation, and samples of the supernatant fluid were analyzed for AFB1 concentration.

High-performance liquid chromatography (HPLC) procedure. Samples of supernatant fluid (0.5 ml) were evaporated to dryness under nitrogen gas. Samples were then resuspended in HPLC-grade methanol (0.5 ml), and 10-μl portions were injected into the HPLC. Sample analysis was undertaken as described previously (10).

Statistical analysis. Results obtained from at least two replicate trials were analyzed for significant differences using Duncan’s Multiple Range Test and General Linear Model (GLM) procedures (Statistical Analysis System, SAS Institute, Cary, N.C.).

RESULTS AND DISCUSSION

Experiments were conducted in an effort to suggest a possible biochemical manner by which F. aurantiacum (approximately 10^11 CFU/ml) degrades AFB1. Maximum degradation was obtained after 48 h when F. aurantiacum cells were incubated in the presence of AFB1 alone (cells incubated in the presence of AFB1 alone are referred to as control in all figures). The addition of 0.1, 1, and 10 mM Ca^2+ increased AFB1 degradation after 16 and 24 h, but the increase was significantly different from the control cells only after 48-h incubation (P ≤ 0.05) (Fig. 1). Similar results were obtained with 0.1, 1, and 10 mM Mg^2+, with significantly higher amount of AFB1 degradation after 48-h incubation (Fig. 1). Control samples incubated with AFB1 in phosphate buffer alone (without cells) and control samples containing Ca^2+ or Mg^2+ and AFB1 in phosphate buffer (without cells) did not result in significant AFB1 degradation.

This increase in AFB1 degradation caused by the addition of divalent cations (Ca^2+ and Mg^2+) could be attributed to the fact that these divalent cations are important cofactors of various dehydrogenases and decarboxylases in glycolysis and in the trichloroacetic acid cycle. Mg^2+ is known to stabilize membranes, maintain structural integrity of proteins and nucleic acids, stabilize ribosomes, act as a cofactor with ATP, and activate enzymes (9, 16). Divalent Ca^2+ plays an important role in the structure and function of several enzymes; for example, Ca^2+ is an important cofactor of γ-carboxyglutamate decarboxylases (14). Chemical decontamination of aflatoxin involves reduction and decarboxylation of aflatoxin with the release of nontoxic products. Mg^2+ and Ca^2+ may play a role in enhancing the enzyme system involved in AFB1 degradation. The divalent cations could be stabilizing the system and thus maintaining the enzyme system in an active form for a longer period than if there were no divalent cation present. Also, since AFB1 is the only source of carbon, as the carbon source gets depleted, the divalent cations may be complexing with either ATP or the enzyme system to enhance the metabolic rate of the cell and thus enhance degradation.

Addition of EDTA to the whole cell preparations increased AFB1 degradation after 24 h without significantly increasing the degradation of AFB1 after 16- or 48-h incubation (Fig. 2). Similarly, the addition of 0.1 and 1 mM...
Figures 3 and 4 show the degradation of AFB1 by \textit{F. aurantiacum} at 30°C in the presence of divalent Ca and EDTA or divalent Mg and EDTA. Error bars represent standard deviations. Control refers to AFB1 degradation by \textit{F. aurantiacum} in the presence of AFB1 alone.

OPT resulted in significantly higher rates of degradation after 24-h incubation (Fig. 2). However, a decrease in AFB1 degradation with the addition of 10 mM OPT was observed after 16- and 24-h incubation. EDTA chelates a wide range of metal ions and is a metalloprotease inhibitor. The increase in AFB1 degradation with EDTA and OPT (0.1 and 1 mM) could be due to the binding of inhibitors (possibly divalent cations other than Mg$^{2+}$ and Ca$^{2+}$), which are interacting with AFB1 in some unknown manner or enhancing the metabolism of the cell to degrade AFB1 more rapidly. The decrease in AFB1 degradation observed when 10 mM OPT was present could be due to an inhibitory effect caused by the high concentration of the chelator blocking some metabolic pathway of the cell or binding of the essential divalent cations.

Addition of divalent Ca$^{2+}$ in the presence of EDTA at 1 mM concentration resulted in no significant difference in AFB1 degradation (Fig. 3). However, the addition of Ca$^{2+}$ in the presence of 1 mM OPT significantly lowered rates of degradation (Fig. 4). The highest degradation occurred with 10 mM Ca$^{2+}$ in the presence of 1 mM OPT, and decreased degradation occurred with 1 mM and 0.1 mM Ca$^{2+}$ in the presence of 1 mM OPT. Addition of Mg$^{2+}$ resulted in significantly lower degradation of AFB1 in the presence of 1 mM EDTA (Fig. 3) and 1 mM OPT (Fig. 4) compared with the whole cells with AFB1 alone (control). These results show that Mg$^{2+}$ is strongly bound by the chelators and is possibly an important cofactor involved in AFB1 degradation by the bacterium. Divalent Ca$^{2+}$ is bound more strongly by OPT and therefore may be made unavailable to the cell, resulting in less AFB1 degradation than was observed when divalent Ca$^{2+}$ alone was added to the cells containing AFB1.

These results demonstrate that the divalent cations Ca$^{2+}$ and Mg$^{2+}$ stimulate AFB1 degradation by \textit{F. aurantiacum} and that binding of these cations by chelators make them unavailable to the cell, thus decreasing AFB1 degradation. Based on these results, it is likely that Mg$^{2+}$ is the cation with the higher stimulatory effect. Divalent Mg$^{2+}$ is an important cofactor and stimulator of the pyruvate dehydrogenase system in the glycolytic pathway and pyruvate decarboxylase. Divalent Mg$^{2+}$ is also known to bind ATP; activate the substrate, transfer acyl groups, and phosphate groups (kinases); and increase reaction rates (9, 16). It is possible that reduction of the furan ring occurs during AFB1 degradation by \textit{F. aurantiacum} via a reductase-dehydrogenase system, even though our results with Mg$^{2+}$ and Ca$^{2+}$ do not provide any conclusive evidence of this reaction. Another possible mechanism of AFB1 degradation could be decarboxylation of the coumarin ring via decarboxylases to give detoxified products that are water soluble and chloroform soluble with the release of gaseous CO$_2$ (12, 15). It is also probable that more than one enzyme is involved in AFB1 degradation. Other enzymes potentially involved in aflatoxin degradation may include cytochrome P-450 monooxygenase enzymes (7) and peroxidases (5). A better understanding of the degradative pathway and enzyme system can be obtained once the end products of AFB1 degradation by \textit{F. aurantiacum} are characterized.

Also, research using cellular fractions and crude preparations of the enzyme extracts with the above divalent cations is essential to identify an enzyme or enzyme system involved in AFB1 degradation. Furthermore, characterization of the purified enzyme or enzyme system responsible for the degradation of AFB1 by this bacterium is necessary to define this degradative pathway. Once the enzyme or enzyme system has been identified, the genes coding for the enzymes can be isolated and incorporated into plasmids and transformed into other organisms to be used in decontamination procedures. Alternatively, the genetic material can be directly incorporated into the plant host to obtain resistant crops. Degradative genes for herbicides have been isolated from degradative organisms and transferred via \textit{Agrobacterium tumefaciens} to produce resistant tobacco varieties and other plant species (3). The recent advances in genetics and recombinant DNA technology should enable...
future research toward the identification of genes and their manipulation for use in practical situations and benefit farmers, processors, and the food industry at large.

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