Aflatoxin B₁ Degradation by Flavobacterium aurantiacum in the Presence of Reducing Conditions and Seryl and Sulfhydryl Group Inhibitors

DORIS H. D’SOUZA AND ROBERT E. BRACKETT*

Center for Food Safety and Quality Enhancement, Department of Food Science and Technology, The University of Georgia, Griffin, Georgia 30223-1797, USA

ABSTRACT

This study was undertaken to determine the effects of reducing conditions (β-cysteine) and seryl (phenylmethylsulfonyl fluoride) and sulfhydryl (divalent cadmium) group inhibitors on aflatoxin B₁ (AFB₁) degradation by Flavobacterium aurantiacum. High-performance liquid chromatography was used to determine AFB₁ concentrations in 72-h cultures of F. aurantiacum. The addition of 0.1, 1, or 10 mM L-cysteine did not have any significant effect on AFB₁ degradation by these cultures after incubation for 4, 24, or 48 h (P > 0.05). The addition of 0.1 mM phenylmethylsulfonyl fluoride did not significantly decrease AFB₁ degradation (P > 0.05), but 1 mM phenylmethylsulfonyl fluoride significantly decreased AFB₁ degradation after 4, 24, and 48 h of incubation (P ≤ 0.05). No significant difference in AFB₁ degradation was obtained with 0.1 mM Cd²⁺ after 4, 24, or 48 h of incubation (P > 0.05). The addition of 1 and 10 mM Cd²⁺ significantly decreased AFB₁ degradation compared with the cells containing AFB₁ alone after 4 and 24 h (P ≤ 0.05). The addition of chelators, 1 mM EDTA and 1 mM o-phenanthroline, did not result in removal of inhibition of AFB₁ degradation observed with 1 and 10 mM Cd²⁺. Higher concentration of chelators (>1 mM) are necessary to overcome the inhibitory effect. Further work on the cellular fractions and/or crude enzyme preparations is necessary to determine if indeed sulfhydryl and seryl groups of the enzymes are involved in AFB₁ degradation (by maintaining either the structure or function of the enzyme).

Elimination and reduction of aflatoxin in foods is a persistent problem of growing concern to the agricultural and food industry (12). Aflatoxin-contaminated food and feeds have led to loss in productivity in farm animals. Moreover, aflatoxins are hazardous because they are carcinogenic, mutagenic, and teratogenic. Aflatoxins are somewhat resistant to normal food processing conditions (12). Effective decontamination of foods and feeds via the traditionally used physical and chemical methods is not economically feasible. In addition, these methods cause losses in nutritional and organoleptic qualities of the food. There is also a risk associated with the handling of hazardous but effective chemicals, such as ammonia, by the operators.

In the 1960s, Ciegler et al. (2) screened more than 1,000 microorganisms and found only a single bacterium, Flavobacterium aurantiacum NRRL B-184, capable of removing aflatoxin from solution. This bacterium can degrade aflatoxin from a wide variety of foods, including peanuts and peanut milk (2, 5). Yet the mechanism by which this bacterium degrades aflatoxin is still under study. This bacterium is capable of degrading aflatoxin B₁ (AFB₁) to water-soluble and chloroform-soluble compounds and gaseous CO₂ (10). Line and Brackett (8) found that higher population of cells (>10¹¹) and older cultures (72 h) are more effective in aflatoxin removal than lower populations or younger cultures (24 or 48 h). They also postulated that AFB₁ removal by F. aurantiacum is a mineralization phenomenon, since additional carbon sources did not affect AFB₁ degradation (9). Results of the recent work by Smiley and Draughon (13) on crude protein extracts from F. aurantiacum suggest that the degradation of AFB₁ by this bacterium may be enzymatic.

The goals of this research were to (i) determine if reducing conditions enhance or inhibit AFB₁ degradation by F. aurantiacum, (ii) determine if a sulfhydryl group plays an important role in AFB₁ degradation, and (iii) determine if serine is an active group involved in AFB₁ degradation. This research aims at understanding the enzymatic involvement in AFB₁ degradation by F. aurantiacum with the hope of purifying the enzyme system. Once the enzyme system has been identified, purified, and characterized, their genes can be cloned. These genes can be transferred into suitable vectors for the overexpression of their proteins and can be used in the detoxification of animal feeds. Alternatively, they can be incorporated directly into plants to produce resistant varieties.

MATERIALS AND METHODS

Cell culture. F. aurantiacum NRRL B-184 was obtained from L. K. Nakamura (U.S. Department of Agriculture, Northern...
Regional Research Center, Peoria, Ill.). The cells were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at 30°C and maintained by transferring at approximately 3-day intervals to fresh broth and tryptic soy agar slants as described earlier (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 L-cysteine, phenylmethylsulfonyl fluoride (PMSF), Cd\(^{2+}\), or Cd\(^{2+}\) and chelators (1 mM EDTA or 1 mM o-phenanthroline [OPT]) added together (Sigma Chemical Co., St. Louis, Mo.). AFB1 (Sigma) was obtained in its crystalline form, resuspended in chloroform, and added to phosphate buffer (10). *F. aurantiacum* cells were grown in 500 ml of tryptic soy broth at 30°C with constant agitation (150 rpm) in a similar manner as described earlier (3). Portions of the culture were serially diluted in 0.1 M phosphate buffer, pH 7.0, and enumerated by surface spreading on tryptic soy agar. Equal portions (10 ml) of the culture were added to 15-ml test tubes, pelleted by centrifugation at 1,800 \(g\) for 10 min, and resuspended in equal volumes of phosphate buffer AFB1 (10 \(\mu\)g/ml); AFB1 (10 \(\mu\)g/ml) and divalent cadmium (0.1, 1, or 10 mM); AFB1 (10 \(\mu\)g/ml) and divalent cadmium (0.1, 1, or 10 mM), and chelator (1 mM EDTA or 1 mM OPT); or AFB1 (10 \(\mu\)g/ml) and PMSF (0.1 or 1 mM) were added to these samples. Controls used included (i) AFB1, (ii) AFB1 and cysteine, (iii) AFB1 and Cd\(^{2+}\), (iv) AFB1 and chelator (v) AFB1, Cd\(^{2+}\), and chelator, and (vi) AFB1 and PMSF in phosphate buffer without the addition of cells. Samples in phosphate buffer with just the effectors (without any added AFB1) were also used as controls. Cells (approximately 10\(^{11}\) CFU/ml) were incubated at 30°C with agitation at 150 rpm for 0, 4, 24, and 48 h and pelleted by centrifugation. Samples of the supernatant fluid were analyzed for AFB1 concentration by high-performance liquid chromatography (HPLC).

**HPLC procedure.** The HPLC procedure used for analysis of AFB1 content was similar to that described by Line and Brackett (8). 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) passed through nylon acrodisc syringe filters (Fisher Scientific, Pittsburgh, Pa.), and 10-\(\mu\)l portions were injected into the HPLC and analyzed.

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

**RESULTS AND DISCUSSION**

The addition of 0.1, 1, and 10 mM L-cysteine to *F. aurantiacum* (approximately 10\(^{11}\) CFU/ml) did not significantly affect AFB1 degradation after 4, 24, or 48 h (Fig. 1). Reducing conditions did not appear to play a role in enhancing or reducing AFB1 degradation by *F. aurantiacum*. Alternatively, L-cysteine may not be an effective reducing agent in AFB1 degradation by this bacterium. Significant increases ... conditions play an important role in AFB1 degradation, as seen with the extracellular cysteine proteinase produced by *Micrococcus* sp. INIA 528 (4). Earlier work by Line (7) showed that 10 mM dithiothreitol, an agent that reduces disulfides, had no significant effect on the ability of *F. aurantiacum* to degrade AFB1. Studies with cellular fractions and crude preparations and with additional reducing agents (such as glutathione and/or \(\beta\)-mercaptoethanol) will also aid in determining if reducing conditions enhance or inhibit AFB1 degradation by this bacterium.

The effect of divalent cadmium was studied to determine if a sulfhydryl group was involved in AFB1 degradation by *F. aurantiacum*. No significant difference in AFB1 degradation was observed in the presence of 0.1 mM Cd\(^{2+}\). Decreased AFB1 degradation was observed using 1 and 10 mM Cd\(^{2+}\) compared with cells containing AFB1 alone after 4 and 24 h (Fig. 2). The addition of 1 mM...
EDTA (Fig. 3) and 1 mM OPT (Fig. 4) did not counteract the inhibition of AFB1 degradation in the presence of 1 and 10 mM Cd$^{2+}$. These higher Cd$^{2+}$ concentrations could be toxic to the cell by blocking the metabolism of the cell and decreasing AFB1 degradation. Cadmium is toxic to most cells because it binds essential sulfhydryl (thiol) groups and makes them unavailable for activity. It is likely that Cd$^{2+}$ is binding the essential sulfhydryl groups involved in AFB1 degradation by *F. aurantiacum* at the higher concentrations (1 and 10 mM) studied. Cadmium also denatures proteins and induces DNA damage that lead to carcinogenic and mutagenic effects (11). On the other hand, microorganisms tolerate cadmium by binding toxic cations to polyphosphates (6). Other mechanisms of cadmium resistance in bacteria include energy-dependent efflux systems, such as in *Staphylococcus aureus*, and reduced transport of the metal into the cells, as seen in *Bacillus subtilis* (1). They also gain resistance to cadmium by harboring plasmids that probably convert the toxic cations to nontoxic or biologically inert forms. This toxic effect of cadmium could have been overcome during 48 h in order that the bacteria survive.

PMSF at concentrations of 0.1 and 10 mM was added to *F. aurantiacum* cells to determine if seryl groups are involved in seryl groups are involved in the enzymatic system of AFB1 degradation. PMSF is a known serine protease inhibitor and causes inactivation by blocking the seryl active group of an enzyme (4). Incubating the cells with 0.1 mM PMSF did not significantly affect AFB1 degradation after 4, 24, and 48 h of incubation (Fig. 5). However, 1 mM PMSF significantly decreased AFB1 degradation after 4, 24, and 48 h. Preliminary data (not shown) revealed an approximately 1.5-log decrease in populations of *F. aurantiacum* cells exposed to 1 mM PMSF for 24 and 48 h. The higher concentration of PMSF may be toxic to the cell itself by inhibiting an important seryl active enzyme and decreasing the metabolism of the cell. Alternatively, 1 mM PMSF may be inhibiting the enzymatic system involved in AFB1 degradation by *F. aurantiacum* by blocking an active seryl group. The seryl group could be playing an important role in maintaining either the structure or the function of the enzyme. Work on the cellular fractions and crude enzymatic preparations is essential to determine conclusively whether the seryl group plays an important role in AFB1 degradation.

The role played by reducing conditions and seryl and sulfhydryl inhibitors on the crude enzymatic preparations is essential to support the preliminary results obtained herein using whole cells. The work on the crude enzymatic preparations should help in the identification, purification, and
characterization of the enzyme system involved in AFB1 degradation, which can be applied in the detoxification of food and feeds and/or obtaining resistant crops.

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