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Research Note

Preliminary Evidence that Degradation of Aflatoxin B_1 by *Flavobacterium aurantiacum* is Enzymatic

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ABSTRACT

The ability of crude protein extracts from *Flavobacterium aurantiacum* to degrade aflatoxin B_1 (AB_1) in aqueous solution was evaluated. Crude protein extracts (800 μg of total protein per ml) degraded 74.5% of AB_1 in solution. An average of 94.5% of AB_1 was recovered after incubation with heat-treated crude protein extracts (800 μg of total protein per ml). DNase I–treated crude protein extracts degraded 80.5% of AB_1 in solution, suggesting that removal of aflatoxin by *F. aurantiacum* is not due to nonspecific binding with the bacterium's genomic DNA. Proteinase K–treated crude protein extracts degraded 34.5% of AB_1, providing evidence that degradation of aflatoxin is linked to a protein that is possibly an enzyme. Solution pH affected the amount of AB_1 degraded by crude protein extracts after 24 h. Maximum degradation was observed at pH 7 (pH levels tested: 5, 6, 7, and 8), with some AB_1 degradation occurring at pH levels as low as 5 and as high as 8. Acidic pH levels were more detrimental to the ability of crude protein extracts to degrade AB_1 than was basic pH. The results of this work indicate that the degradation of AB_1 by *F. aurantiacum* may be enzymatic.

The first evidence of the ability to degrade aflatoxin B_1 (AB_1) by live cells of *Flavobacterium aurantiacum* was reported by Ciegler et al. *(2)*. One thousand microorganisms, including yeasts, molds, and bacteria, were screened. Only *F. aurantiacum* was shown to irreversibly remove AB_1 from both solid and liquid media. Log-phase cells of *F. aurantiacum* were reported to remove 30% of AB_1 after 19 h and 74% of AB_1 after 44 h. No further degradation of AB_1 was observed during the 68-h incubation *(2)*. Stationary-phase cells were reported to remove 18% of AB_1 after 16 h, 41% of AB_1 after 41 h, and 100% of AB_1 after 88 h *(2)*. Heat-inactivated (autoclaved) cells were not able to remove AB_1 from the test medium *(2)*. Lillehoj et al. *(6)* reported that an inoculum level of 11.7 $\log_{10}$ CFU/ml degraded 49% of AB_1 after 5 min and that an inoculum level of 12.6 $\log_{10}$ CFU/ml degraded 73% of AB_1 after the same amount of time. The optimum temperature for AB_1 degradation by *F. aurantiacum* has been reported to be 35°C *(6)*. The optimum pH for AB_1 degradation (1.3 μg/h) has been reported to be 6.75 *(6)*. At pH 5.0, the rate of removal of AB_1 was 0.6 μg/h, and at pH 8.0, the rate of removal of AB_1 was 0.8 μg/h *(6)*. The degradation of AB_1 in food products by *F. aurantiacum* has been reported *(2, 4)*. Ciegler et al. *(2)* reported that cells of *F. aurantiacum* removed 100% of AB_1 from milk after 2 h, 100% of AB_1 from vegetable oil after 3 h, and 100% of AB_1 from peanut butter after 4 h. *F. aurantiacum* was also shown to remove AB_1 from ground soybeans, corn, and peanuts *(2)*. Hao and Brackett *(4)* reported that *F. aurantiacum* (9 $\log_{10}$ CFU/ml) removed 82% of AB_1 from non-defatted peanut milk, 51% of AB_1 from partially defatted peanut milk, and 40% of AB_1 from phosphate buffer after 24 h. *F. aurantiacum* has also been shown to remove 99% of aflatoxin M_1 from solution after 60 h *(8)* and 100% of aflatoxin G_1 from solution after 240 min *(7)*. Before 1994, the exact fate of AB_1 in the presence of *F. aurantiacum* was not known. Line et al. *(10)*, using ^{14}C-labeled AB_1, determined the amount of radioactivity in both the organic and aqueous phases during chloroform extraction after reaction of AB_1 with live cells of *F. aurantiacum*. It was reported that only 24% of the radioactivity remained in the organic phase after 6 h *(10)*. Controls, containing no *F. aurantiacum*, retained all the radioactivity in the chloroform phase. This provided the first evidence that AB_1 was being degraded by the bacterium. In a subsequent study, Line and Brackett *(9)* reported that the addition of another nutrient source (trypticase soy broth) did not affect the removal of AB_1, indicating that AB_1 was probably being degraded through a secondary metabolic pathway.

No information is currently available detailing the mechanism of degradation of AB_1 by *F. aurantiacum*. It is possible that removal of AB_1 is due to nonspecific binding to either the cell wall or other structures within the bacterium or to an enzyme or enzyme system. The purpose of this study was to investigate the degradation of AB_1 to determine whether the mechanism was linked to a protein, which may indicate enzymatic processes, by examining the effects of proteinase K, DNase I, and pH on the degradation of AB_1 by crude protein extracts from *F. aurantiacum*.

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MATERIALS AND METHODS

Cultures. *F. aurantiacum,* strain NRRL B-184 (U.S. Department of Agriculture–Agricultural Research Service, Northern Regional Research Center, Peoria, Ill.), was grown using brain-heart infusion broth (Difco Laboratories, Detroit, Mich.) at 25°C in an orbital shaker incubator. Cells were transferred after 12 h to fresh broth and incubated an additional 24 h. Cells were pelleted by centrifugation (6,000 × *g*), and the spent medium was discarded. Cells were then suspended in sterile brain-heart infusion broth containing 50% (vol/vol) sterile glycerol. Glycerol stocks were stored at −70°C throughout the study.

Preparation of crude protein extracts. *F. aurantiacum* (stock culture) was inoculated into 100 ml of brain-heart infusion broth and grown for 24 h at 25°C using an orbital shaker incubator. Cells (5 μl) were transferred to 1 liter of fresh brain-heart infusion broth and incubated for 48 h at 25°C using an orbital shaker incubator. After 48 h of incubation, cells were harvested by centrifugation (6,000 × *g* for 10 min), and the cell pellets were resuspended using 100 ml of sterile buffer (50 mM Tris-HCl, pH 7.2, 0.1% [vol/vol] Triton X-100, 200 mM NaCl, 10% [wt/vol] sucrose, and 100 mM Na-EDTA). The cells were incubated for 12 h at 4°C without shaking to weaken the outer cell membrane. After the 12-h incubation, cells were pelleted by centrifugation at 6,000 × *g* for 10 min, and the supernatant fluid was discarded. The cells were resuspended using 25 ml of fresh buffer (50 mM Tris-HCl, pH 7.2, 50 mM EDTA, 200 mM NaCl, 10% [wt/vol] sucrose, and 0.1% [vol/vol] Triton X-100). Lysozyme (E.C. 3.2.1.17, Sigma Chemical Co., St. Louis, Mo.) was added at a concentration of 0.5 mg/ml, and the cell-lysozyme suspension was incubated for 24 h at 4°C. After cell lysis, cellular debris was removed by centrifugation a 20,000 × *g* at 4°C. The supernatant fluid (crude protein extract) was collected and stored on ice. The crude protein extract was then equally divided into three portions (approximately 8 ml each). The total protein concentration was determined and adjusted to a final concentration of 800 μg/ml using the same buffer as previously described. One portion was maintained on ice, another was heated in a boiling-water bath (in a screw-capped test tube) for 15 min, and the third was treated with proteinase K (0.1 mg/ml) (Roche Molecular Biochemicals, Indianapolis, Ind.) for 6 h at 4°C. Crude protein extracts treated with DNase I (Roche) were prepared similarly to the others except that the lysis buffer composition was changed to 50 mM Tris-HCl, 50 mM $MgCl_2$, 200 mM NaCl, 10% (wt/vol) sucrose, 0.1% (vol/vol), and Triton X-100 because magnesium is a required cofactor for DNase I. Total protein concentration was determined and adjusted to 800 μg/ml using the buffer described above. DNase I was added to crude protein extracts at a level 100 μg/ml and allowed to react for 6 h at 4°C. The crude protein extract and the heat-inactivated, proteinase K–treated, and DNase I–treated crude protein extracts were studied for the ability to remove AB_1 from aqueous solution.

Degradation of AB_1 by crude protein extracts. AB_1 (20 μg, from 1,000-ppm stock in acetonitrile) was added to each test tube (20 by 100 mm, screw capped). The test tubes were heated to 50°C under N_2 to evaporate the acetonitrile. The tubes were then cooled (5 min) on ice. Crude protein extract or heat-treated, proteinase K–treated, or DNase I–treated crude protein extract was then added to the appropriate test tubes. The crude protein extract–aflatoxin suspensions were then incubated at 30°C, without shaking, in the dark for 24 h.

Extraction of residual AB_1. After incubation of AB_1 with crude protein extracts or treated crude protein extracts, the reaction was terminated by the addition of 5 ml of high-pressure liquid chromatography–grade chloroform. The reaction mixture was mixed in a vortex mixer for 1 min, and then the contents were added to a 125-ml separating flask. The test tubes were rinsed with an additional 5 ml of chloroform, which was subsequently added to the appropriate separating flask. An additional 15 ml was added to each separating flask, and the flasks were shaken for 2 min. The organic (bottom) phase was collected through sodium sulfate into a 125-ml round-bottom flask. The extraction was repeated again with the addition of 25 ml of chloroform and collected into the appropriate round-bottom flask. The chloroform was then completely evaporated using a Rotavapor-R rotary evaporator (Buchi, Bern, Switzerland) at a temperature of 55°C. The remaining aflatoxin was then dissolved in 3 ml of high-pressure liquid chromatography–grade acetonitrile and analyzed immediately by spectrophotometry.

Quantitative analysis of residual AB_1. After extraction of residual AB_1, the amount remaining after reaction with crude protein extracts or treated crude protein extracts was determined using a Hewlett Packard 8452 diode array spectrophotometer (Hewlett-Packard Co., Palto Alto, Calif.) by a modified method described by Nabney and Nesbitt *(11)*. Absorbance values were measured (λ = 363 nm), and concentrations were determined by comparing values to standard curves prepared from known concentrations of AB_1. The equation for a straight line ($Y = mX + b$), obtained using least-squares analysis (Axum, Cambridge, Mass.), was determined for the standard curve and used to determine unknown concentrations of remaining AB_1. Each experiment was replicated three times. The values reported are the average of the three replicates.

Determination of crude protein extract concentration. Protein concentration for crude protein extracts and treated crude protein extracts was determined using BioRad Coomassie blue staining *(1)*. For each extract tested, 1 ml of concentrated dye was diluted with 4 ml of distilled, deionized water for a total volume of 5 ml. Crude protein extracts (100 μl) were added to the diluted dye. The dye-protein mixture was incubated for 5 min at 25°C so that reaction times were standardized. Absorbance values were measured (λ = 595 nm). A standard curve was produced using known concentrations of bovine serum albumin using the same procedure. The concentration of crude protein extracts was calculated from the equation for a straight line (given above), obtained using least-squares analysis, determined from the standard curve.

Effect of pH on AB_1 degradation. The ability of crude protein extracts to degrade AB_1 at different pH levels was evaluated. One liter of brain-heart infusion broth was inoculated with 5 μl of a 12-h culture of *F. aurantiacum.* Cells were grown for 48 h at 25°C using an orbital shaker incubator. Cells were harvested by centrifugation at 6,000 × *g* for 10 min. Cell pellets were resuspended using 50 ml of buffer (50 mM Tris-HCl, 50 mM EDTA, 200 mM NaCl, 10% [wt/vol] sucrose, 0.1% [vol/vol], and Triton X-100, pH 7.2). The cells were incubated for 12 h at 4°C without shaking to weaken the outer cell membrane of the bacterium. After 12 h of incubation, cells were pelleted as described above and resuspended using fresh buffer (described above). Lysozyme was added at a concentration of 0.5 mg/ml. Cells were lysed for 24 h at 4°C. The supernatant fluid (crude protein extract) was clarified by centrifugation at 20,000 × *g* for 30 min. Buffers, for pH analysis, were prepared as follows: pH 5.0, 50 mM sodium acetate/acetic acid; pH 6.0, 50 mM $HPO_4^{2-}/H_2PO_4^-$; pH 7.0, 50 mM Tris-HCl; and pH 8.0, 50 mM Tris-HCl. The supernatant fluid

TABLE 1. *Removal of AB_1 from solution by crude protein extract (800 μg of total protein per ml) from* F. aurantiacum, *heat-treated crude protein extract, proteinase K–treated crude protein extract, and DNase I–treated crude protein extract during 24 h of incubation at 30°C*

Extract	AB_1 concentration (μg) Initial	Final[a]	% Removal	SE[b]
Control	20	19.2 A	4.0	1.1
Crude	20	5.1 C	74.5	2.9
Heat-treated	20	18.9 A	5.5	1.0
Proteinase K-treated	20	13.1 B	34.5	3.1
DNase I-treated	20	3.9 C	80.5	1.1

[a] Means followed by different letters are significantly different ($P < 0.05$).
[b] Standard error of the mean determined from three replications. Control reaction performed by incubating 20 μg of AB_1 in 50 mM Tris-HCl, pH 7.2, without addition of crude protein extract.

was divided into 5-ml aliquots, which were then added to Spectrum dialysis tubing (Medical Industries, Inc., Los Angeles, Calif.), which had a molecular weight cutoff of 3,000 Da. Buffers were exchanged by dialysis in excess buffer (of appropriate pH) for 6 h. After equilibration, the final pH was checked and minor adjustments made using the appropriate acid or base as needed. Total protein concentration was determined and adjusted to 960 μg/ml using the appropriate buffer as described above. The crude protein extracts, adjusted to pH 5, 6, 7, or 8, were evaluated for their effectiveness at degrading AB_1 in aqueous solution.

RESULTS

Crude protein extracts and treated crude protein extracts. Crude protein extracts (800 μg of total protein per ml) degraded an average of 74.5% of AB_1 (initial concentration of 20 μg) in aqueous solution after 24 h of incubation at 30°C (Table 1). Heat treatment (boiling for 15 min) resulted in only 5.5% of AB_1 being degraded by crude protein extract (800 μg of total protein per ml) (Table 1). This observation confirms previous observations *(2, 10)*. Ciegler et al. *(2)* reported that heat-treated (autoclaved) cells of *F. aurantiacum* failed to irreversibly remove AB_1 from aqueous solution. In addition, Line et al. *(10)* reported that heat-treated *F. aurantiacum* failed to convert the normally nonpolar soluble AB_1 to the more water-soluble products that was observed by live cells. Heat treatment of crude protein extracts does not conclusively prove that degradation of AB_1 is due to an enzyme because other cellular constituents and structures are also heat labile. Proteinase K–treated crude protein extracts (800 μg of total protein per ml before the addition of proteinase K) degraded approximately 34.5% of AB_1 (initial concentration of 20 μg). Proteinase K is a nonspecific protease and will react with all proteins present in the crude extracts. Other proteins present in the crude protein extract can also serve as substrate for proteinase K, so 100% inactivation of the degradation of AB_1 is not expected. The diminished AB_1-degrading activity of proteinase K–treated crude protein extracts indicates that degradation of AB_1 is due to a protein and perhaps an enzyme. DNase I–treated crude protein extracts (800 μg of total protein per ml before the addition of DNase I) decreased AB_1 by 80.5% (initial concentration of 20 μg). DNase I treatment was used to determine whether removal of AB_1 was due to nonspecific binding to the bacterial genomic DNA. DNase I is an enzyme that degrades both single- and double–stranded DNA producing 3′-hydroxyl oligonucleotides. Because AB_1 degradation did not decrease after crude protein extracts were treated with DNase I, it is evident that removal of AB_1 by *F. aurantiacum* is not occurring through binding to the bacterium's chromosomal DNA. This observation is confirmed by toxicity studies *(3)*, which indicate that epoxidation of AB_1 is necessary before binding to DNA can occur. Because bacteria lack microsomal enzymes, epoxidation would not be expected, preventing binding to bacterial chromosomal DNA. The slightly higher amount of degradation observed for the DNase I–treated crude protein extracts over the nontreated crude protein extracts may be attributable to the removal of the genomic DNA, which may compete for binding, through temporary nonspecific interaction between the DNA and AB_1, preventing the enzyme and AB_1 from contacting one another.

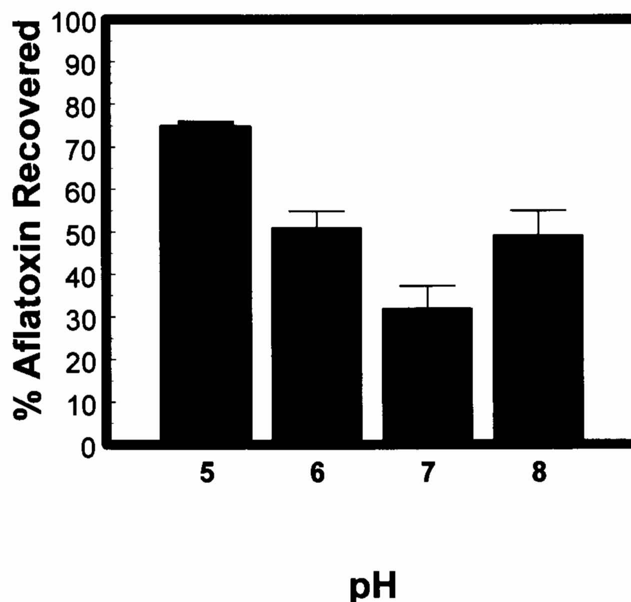

FIGURE 1. *AB_1 degradation by crude protein extracts (960 μg of total protein per ml) from* F. aurantiacum *at various pH levels during 24 h of incubation at 30°C. Error bars reflect the amount of variation associated with the three replications.*

Effects of pH on AB_1 *degradation.* The effect of pH on the degradation of AB_1 by crude protein extracts from *F. aurantiacum* was evaluated. Crude protein extracts (960 μg of total protein per ml) degraded approximately 25% of AB_1 (20 μg) at pH 5, 50% at pH 6, 70% at pH 7, and 50% at pH 8 (Fig. 1). The maximum amount of AB_1 degradation for crude protein extracts occurred at a neutral pH. The relationship between degradation of AB_1 and pH is typical of an enzymatic reaction. Enzymes have an optimum pH range for maximal activity. At pH values above and below the optimum, enzymatic activity decreases *(5)*. Enzymes are proteins composed of amino acids, many of which can act

as weak acids or weak bases in enzyme-catalyzed reactions. The decrease in enzyme activity observed when pH is outside the optimum range is usually due to the ionization of a critical amino acid residue within the catalytic site *(5)*. At times, ionization of residues outside the catalytic site can lead to overall conformational changes, which can also decrease activity. Lillehoj et al. *(6)* reported that the rate of AB_1 removal by live cells of *F. aurantiacum* was maximum at pH 6.75, which correlates well with the results of this study. No previous research has been reported on the effects of pH on the ability of crude protein extracts to degrade AB_1.

CONCLUSIONS

Crude protein extracts demonstrated the ability to degrade aflatoxin B_1. The results from the proteinase K– and DNase I–treated crude protein extracts and the results from the pH study suggest that the degradation of AB_1 is linked to a protein that has characteristics typical of enzymes. The results from this study also suggest that the component within the crude protein extract of *F. aurantiacum*, responsible for the degradation of AB_1, might be stable in a purified form when isolated and used in the food industry as a means of removing aflatoxins from contaminated foods. Although the maximum amount of AB_1 degradation was observed to occur at pH 7, 22 to 45% degradation was still observed at the other pH values tested, making it applicable to a wide range of foods.

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