Identification of a Variant of *Fusarium proliferatum* That Hydrolyzes Fumonisin B_1

S. E. Keller* and T. M. Sullivan

National Center for Food Safety and Technology, U.S. Food and Drug Administration, 6502 South Archer Avenue, Summit-Argo, Illinois 60501

Fumonisins are mycotoxins produced primarily by *Fusarium moniliforme* and *Fusarium proliferatum* in corn. Normally, the fumonisins display considerable chemical stability at temperatures up to 100 °C at neutral pH and are stable in a pH range of 4−10 at ambient temperature. Fumonisin B_1 (FB_1) can be produced in bioreactors using *F. proliferatum* (M5991). Recently, some bioreactor-grown cultures of *F. proliferatum* were found to rapidly hydrolyze FB_1. Subcultures from stock frozen from these FB_1-degrading variants continued to degrade FB_1 when grown under the same bioreactor conditions. The initial hydrolysis was catalyzed by an esterase and resulted in the appearance of the aminopentol backbone (AP_1) of FB_1. A cell-free extract retaining the FB_1 hydrolytic activity could be obtained by extraction of cell paste with 10% Triton X-100. Activity could be destroyed by heating to 60 °C for 5 min or by the addition of acetonitrile.

Keywords: *Fusarium proliferatum; fumonisin, esterase*

INTRODUCTION

Fumonisins are a group of mycotoxins believed to be secondary metabolites produced predominantly by *Fusarium moniliforme*, *Fusarium proliferatum*, and *Fusarium nygamai*. They have been implicated as the causative agents of leukoencephalomalacia (LEM) in horses and pulmonary edema syndrome (PES) in pigs (Harrison et al., 1990; Kellerman et al., 1990; Ross et al., 1990; Wilson et al., 1992). In addition, fumonisins have been associated with an increased incidence of esophageal cancer (Sydenham et al., 1990). Fumonisins were first isolated and characterized from cultures of *F. moniliforme* and subsequently determined to occur in corn worldwide (Gelderblom et al., 1988; Marasas, 1995).

Despite fumonisins' toxicity to animals and their possible role in esophageal cancer, little is known regarding production by *Fusarium* species. *Fusarium* species are ubiquitous on corn, and even visibly healthy corn has been found to contain high levels of fumonisin (Bacon et al., 1992). Generally, fumonisins are produced for study in corn-based solid media (Leslie et al., 1992a,b; Nelson et al., 1991, 1992, 1993; Thiel et al., 1991; Ross et al., 1990). On corn-grown cultures fumonisins generally appear after ≈2 weeks of incubation and, depending on growth conditions, continue to increase up to at least 4 weeks (Alberts et al., 1990; Nelson et al., 1994). Decreases in fumonisin B_1 (FB_1) production were noted after 13 weeks (Nelson et al., 1994). In liquid cultures fumonisin can appear in as little as 3 days, with production continuing for at least 30 days (Blackwell et al., 1994; Jackson and Bennett, 1990; Keller and Sullivan, 1995). Although the ability to metabolize fumonisin has been observed in the yeast *Exophiala spinifera*, breakdown by the producing organism has not been demonstrated (Duvick et al., 1994).

Recently, we found evidence indicating that fumonisin may be an intermediate which is metabolized by the producing organism. In some liquid cultures of *F. proliferatum* (M5991) originally grown for the production of FB_1, the FB_1 was not stable as was anticipated. The FB_1 that was produced was hydrolyzed to its C_{22} aminopentol (AP_1) backbone. After extensive cultivation, the AP_1 also disappeared. Its degradative products have not been identified.

MATERIALS AND METHODS

Culture Methods. Freeze-dried *F. proliferatum* (M5991) culture was obtained from the Fusarium Research Center at the Pennsylvania State University. Working stock cultures were obtained by suspending freeze-dried culture in deionized water followed by inoculation into media modified from Jackson and Bennett (1990) as follows: 90 g L^{-1} glucose, 3.5 g L^{-1} $(NH_4)_2SO_4$, 1.0 g L^{-1} KH_2PO_4, 1.0 g L^{-1} K_2HPO_4,, 0.3 g L^{-1} $MgSO_4$, 0.4 g L^{-1} $CaCl_2$, 16 mg L^{-1} $MnSO_4$. Vitamins were filter sterilized and added after autoclaving as follows: 1 mg L^{-1} each of thiamin, riboflavin, pantothenoate, niacin, pyridoxamine, and thiotic acid and 100 μg L^{-1} each of folic acid, biotin, and B_{12}. Shake flask cultures (100 mL in a 500 mL baffled Erlenmeyer flask) were placed at 25 °C on a rotary shaker set at 200 rpm and incubated for 7 days. After 7 days, the cultures were centrifuged and resuspended in fresh media with 50% glycerol. Additional working stock was also produced by first harvesting spores from *F. proliferatum* grown on solid media and then growing isolated colonies from single spores prior to transfer to shake flask for further cultivation as indicated. For growth studies, ≈2 mL of frozen stock was cultured in Jackson and Bennett medium as before and then transferred to additional Jackson and Bennett medium for growth studies. For growth and production studies, two 2-L Braun glass stirred-jar reactors and one 100-L and two 10-L stainless steel stirred reactors were used (Braun Biotech, St. Louis, MO). Standard conditions used in bioreactor studies were those described by Keller and Sullivan (1997). Additional growth and production studies were also conducted in 500 mL baffled Erlenmeyer flasks using conditions described by Keller and Sullivan (1997).

* Author to whom correspondence should be addressed [telephone (708) 728-4140; fax (708) 728-4177; e-mail skeller@charlie.cns.iit.edu or SEK@vm.cfsan.fda.gov].

S0021-8561(98)00031-4
Published on Web 05/30/1998

Chemical Analysis. For analysis of fumonisin and its hydrolyzed AP_1 backbone in growing cultures, the cultures were first filtered using a 0.45 μm syringe filter (Gelman Sciences, Ann Arbor, MI) to remove cells. No other purification of broth was required prior to analysis. The filtrate was then diluted to contain between 2 and 10 μg/mL FB_1 for analysis. FB_1 and AP_1 standards were kindly provided by Dr. R. Eppley, FDA, CFSAN, Washington, DC. FB_1 and AP_1 levels were determined using the HPLC method of Shephard et al. (1990).

Enzyme Anaylsis. Cell paste was obtained by harvesting bioreactors with tangential flow 0.2 μm Prostak ultrafiltration unit (Millipore Co., Bedford, MA). Cell paste used for enzyme activity was extracted at 25 °C by shaking for 30 min with Triton X-100 (Fisher Scientific, Pittsburgh, PA; 10% final Triton X-100, v/v, in 0.1 M sodium citrate buffer, at pH 5.0). The crude cell-free extract was then concentrated using a Minitan tangential flow filtration system (Millipore) with a 10 000 MW cutoff membrane filter. Protein concentration in the cell-free extract was determined with the Lowry reaction (Sigma Chemical Co., St. Louis, MO) using bovine serum albumin (BSA) as the standard. Enzyme activity was measured by adding a known amount of FB_1 in 0.1 mL of deionized H_2O to 0.1 mL of culture filtrate and 0.3 mL of sodium citrate buffer (0.1 M, at pH 5.0) at 30 °C. The reaction was stopped by removing 0.05 mL and adding it to 0.95 mL of acetonitrile/H_2O (50:50). Disappearance of FB_1 was quantified over time using HPLC as indicated above. Units were defined as the loss of 1 μg of FB_1/min. Trypsin and proteinase K were obtained from Sigma. Each was added to the crude cell-free extract at a final concentration of 1 mg/mL. The mixture was held at ambient temperature for 24 h and then assayed for FB_1-degrading activity as indicated above.

RESULTS AND DISCUSSION

Jackson et al. (1996) studied the effect of temperature and pH in an aqueous system on the nonenzymatic hydrolysis of FB_1. Although the lowest pH examined was 4.0, at 100 °C, no degradation was observed even after 2 h. Temperatures in excess of 100 °C were required before degradation was observed. During growth and production of FB_1 either in shake flasks or in bioreactors, the temperature was controlled at 25 °C (Keller and Sullivan, 1997). In shake flasks, the pH may drop below 2.0; however, in bioreactors, the pH is controlled at 3.5. Therefore, little, if any, chemical hydrolysis of FB_1 is likely to occur under the conditions used for the growth of *F. proliferatum*. In previous shake flask studies, no loss of FB_1 was found, even after 60 days of incubation, regardless of final pH (Keller and Sullivan, 1997). In contrast, during some experiments examining the production of FB_1 in bioreactors, FB_1 was seen to sharply disappear after an initial accumulation (Figure 1). Not all cultures from the original stock culture of *F. proliferatum* grown in bioreactors displayed FB_1-degrading activity. Of 11 bioreactor cultures (2, 10, and 100 L), each producing at least 500 ppm of FB_1, only 3 showed degradative activity. All bioreactor cultures were routinely checked for contamination, but none was detected in either type of culture. Furthermore, growth, glucose consumption, and oxygen levels were not different in either type of culture. Stock frozen from cultures showing FB_1 degradative activity continued to show activity when grown under the same conditions in bioreactors. The FB_1-degrading cultures were examined for purity both microscopically and by further cultivation (liquid and solid media for single colonies), and no contaminating organisms were found.

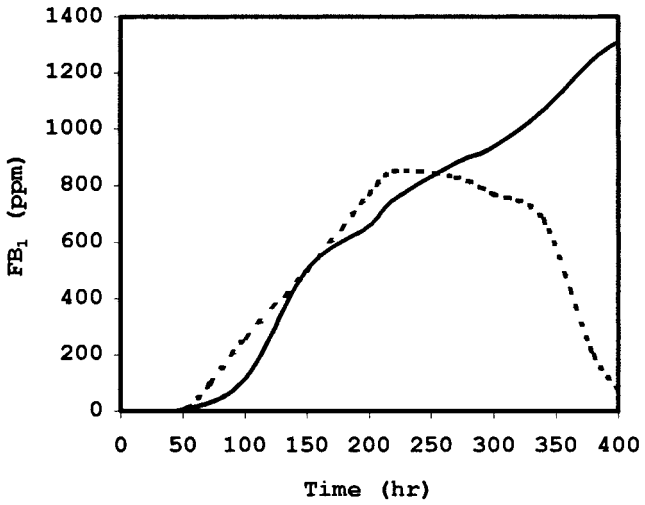

Figure 1. FB_1 concentrations in two *F. proliferatum* bioreactor experiments: (—) bioreactor culture without esterase activity; (- - -) bioreactor culture with esterase activity.

Subsequent shake flask grown cultures producing FB_1, whether from the original stock or subcultured from bioreactor cultures with FB_1-degrading activity, never showed detectable FB_1 losses, regardless of the pH of cultivation. Differences in oxygenation are substantial in shake flask and bioreactor-grown cultures and can greatly influence the level of FB_1 produced (Keller and Sullivan, 1997). In previous studies, well-aerated conditions were linked to higher FB_1 production. As a consequence, considerably more FB_1 can be produced in bioreactors. Therefore, the differences in oxygenation and the lower FB_1 concentrations may account for the inability to observe FB_1 degradation in shake flasks.

As the FB_1 concentration during bioreactor cultivation of the variant cultures decreased, a transitory increase in AP_1 concentration also appeared. However, no partially hydrolyzed FB_1 was ever observed in cultures showing FB_1 degradation. AP_1 did not accumulate in cultures without FB_1 losses. To obtain AP_1 from FB_1, the ester linkages at the C-14 and C-15 carbons must be hydrolyzed. This type of reaction would indicate the presence of a carboxylic esterase. To determine if an esterase from *F. proliferatum* was responsible for the hydrolysis of FB_1 to AP_1, cells (*F. proliferatum* variant, grown in a 100 L bioreactor) were harvested, extracted, and concentrated. After harvesting, all activity was found in the retentate. Extraction with Triton X-100 removed ≈80% of the activity in the cell paste. After concentration of the extracted material, FB_1-degrading activity was found in the retentate but not in the filtrate. The final concentrated crude cell-free preparation contained ≈6.0 mg/mL protein. The cell-free extract was added to FB_1 in 0.1 M sodium citrate buffer, pH 5.0, at 30 °C and sampled at 10 min intervals to determine the apparent specific activity.. The estimated apparent specific activity for the esterase was 1.3 units/mg of protein at pH 5.0 and 30 °C. HPLC examination showed an increase in a peak corresponding to AP_1 as the added FB_1 decreased (Figure 2). The concentration of AP_1 corresponded to the loss of FB_1 in approximately a 1:1 ratio. Although the reaction with FB_1 was unaffected when the crude concentrate was treated with trypsin or proteinase K, the reaction was stopped completely with heat treatment (5 min at 60 °C) or the addition of 50% methanol or acetonitrile.

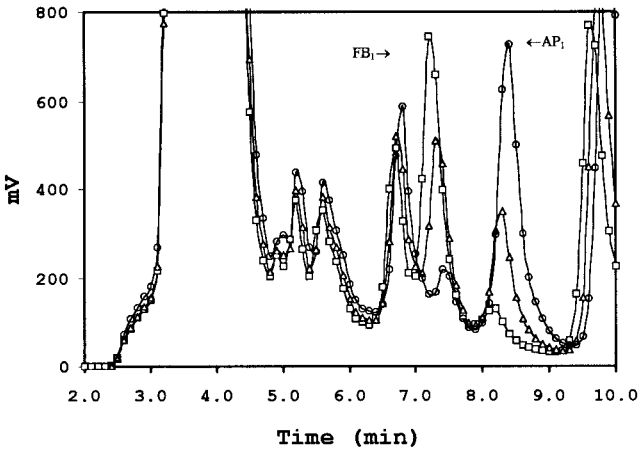

Figure 2. HPLC chromatograph showing the disappearance of FB_1 at 7.4 min elution time and the appearance of AP_1 at 8.4 min elution time in a typical assay using enzyme extracted from *F. proliferatum*. Reaction incubation times were (□) t = 0, (△) t = 20 min, and (○) t = 60 min.

In the reaction with the cell-free extract, no further degradation or loss of AP_1 was observed. This is in contrast to observations with the whole culture, in which the accumulation of AP_1 appears transitory, albeit after several days. The cause of loss or further degradation of AP_1 in the bioreactors is unknown and may be due to causes other than biological.

CONCLUSIONS

In the literature, there is some notation of decreases in FB_1 concentration during extensive cultivation of the producing cultures (Nelson et al., 1994). However, no explanation for these decreases is given and, in general, FB_1 appears to be stable once produced. In addition, the lack of correlation of FB_1 levels to contaminated levels in corn is an anomaly (Bacon et al., 1992). This anomaly can be explained by the presence of enzymes to biologically degrade fumonisins. Such enzymes are impossible to detect in experiments in which solvents are used to extract and quantitate fumonisin because the solvents denature the protein. In this study, a variant of *F. proliferatum* is described that can break down FB_1. The first enzyme in this pathway appears to be an esterase, which hydrolyzes FB_1 to AP_1. It is unlikely that a novel enzyme would abruptly appear in any organism. Therefore, it is probable that the degradation of FB_1 is the result of deregulation or alteration of an enzyme normally produced by the organism. Although the disappearance and ultimate fate of the AP_1 were not investigated, the subsequent loss suggests the presence of other degradative enzymes.

ACKNOWLEDGMENT

We thank Steven M. Gendel and Walter Hargraves of the U.S. Food and Drug Administration for critical evaluation and discussion.

LITERATURE CITED

Alberts, J. F.; Gelderblom, W. C. A.; Theil, P. G.; Marasas, W. F. O.; Van Schalkwyk, D. J.; Behrend, Y. Effects of temperature and incubation period on production of fumonisin B_1 by *Fusarium moniliforme*. *Appl. Environ. Microbiol.* **1990**, *56* (6), 1729−1733.

Bacon, C. W.; Bennett, R. M.; Hinton, D. M.; Voss, K. A. Scanning electron microscopy of Fusarium moniliforme within asymptomatic corn kernals and kernals associated with equine leukoencephalomalacia. *Plant Dis.* **1992**, *76* (2), 144−148.

Blackwell, B. A.; Miller, J. D.; Savard, M. E. Production of carbon 14-labeled fumonisin in liquid culture. *J. AOAC Int.* **1994**, *77* (2), 506−511.

Duvick, J.; Rood, T.; Grant, S. Isolation of fumonisin-metabolizing fungi from maize seed. *Int. Mycol. Congr. 5th* **1994**, 56 (Abstr.)

Gelderblom, W. C. A.; Jaskiewicz, K.; Marasas, W. F. O.; Thiel, P. G.; Horak, R. M.; Vleggaar, R.; Kriek, N. P. J. Fumonisins—novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. *Appl. Environ. Microbiol.* **1988**, *54*, 1806−1811.

Harrison, L. R.; Colvin, B.; Green, J. T.; Newman, L. E.; Cole, J. R. Pulmonary edema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme*. *J. Vet. Diagn. Invest.* **1990**, *2*, 217−221.

Jackson, L. S.; Hlywka, J. J.; Senthil, K. R.; Bullerman, L. B.; Musser, S. M. Effects of time, temperature, and pH on the stability of fumonisin B_1 in an aqueous model system. *J. Agric. Food Chem.* **1996**, *44*, 906−912.

Jackson, M. A.; Bennett, G. A. Production of fumonisin B_1 by *Fusarium moniliforme* NRRL 12616 in submerged culture. *Appl. Environ. Microbiol.* **1990**, *56* (8), 2296−2298.

Keller, S. E.; Sullivan, T. M. Liquid culture methods for the production of fumonisin. In *Fumonisin in Food*; Jackson, L. S., DeVries, J. W., Bullerman, L. B., Eds.; Plenum Press: New York, 1995; pp 205−212.

Keller, S. E.; Sullivan, T. M. Factors affecting the growth of *Fusarium proliferatum* and the production of fumonisin B_1. *J. Ind. Microbiol.* **1997**, *19*, 305−309.

Kellerman, T. S.; Marasas, W. F. O.; Thiel, P. G,; Gelderblom, W. C. A.; Cawood, M. E.; Coetzer, J. A. W. Leukoencephalomalacia in two horses induced by oral dosing of fumonisin B_1. *Onderstepoort J. Vet. Res.* **1990**, *57*, 269−275.

Leslie, J. F.; Doe, F. J.; Plattner, R. D.; Shackelford, D. D.; Jonz, J.; Fumonisin B_1 production and vegetative compatibility of strains from *Gibberella fujikuroi* mating population "A" (*Fusarium moniliforme*). *Mycopathologia* **1992a**, *117*, 37−45.

Leslie, J. F.; Plattner, R. D.; Desjardins, A. E.; Klittich, C. J. R. Fumonisin B_1 production by strains from different mating populations of *Gibberella fujikuroi* (*Fusarium* section Liseola). *Mycotoxicology* **1992b**, *82* (3), 341−345.

Marasas, W. F. O. Fumonisins: History, worldwide occurrence and impact. In *Fumonisin in Food*; Jackson, L. S., DeVries, J. W., Bullerman, L. B., Eds.; Plenum Press: New York, 1995; pp 1−17.

Nelson, P. E.; Plattner, R. D.; Schackelford, D. D.; Desjardins, A. E. Production of fumonisins by *Fusarium moniliforme* strains from various substrates and geographic areas. *Appl. Environ. Microbiol.* **1991**, *57* (8), 2410−2412.

Nelson, P. E.; Plattner, R. D.; Schackelford, D. D.; Desjardins, A. E. Fumonisin B_1 production by *Fusarium* species other than *F. moniliforme* in section Liseola and by some related species. *Appl. Environ. Microbiol.* **1992**, *58* (3), 984−989.

Nelson, P. E.; Desjardins, A. E.; Plattner, R. D. Fumonisins, mycotoxins produced by *Fusarium* species: biology, chemistry, and significance. *Annu. Rev. Phytopathol.* **1993**, *31*, 233−252.

Nelson, P. E.; Huba, J. J.; Ross, P. F.; Rice, L. G. Fumonisin production by *Fusarium* species on solid substrates. *J. AOAC Int.* **1994**, *77* (2), 522−525.

Ross, P. F.; Nelson, P. E.; Richard, J. L.; Osweiler, G. D.; Rice, L. G.; Plattner, R. D.; Wilson, T. M. Production of fumonisins by *Fusarium moniliforme* and *Fusarium proliferatum* isolates associated with equine leukoencephalomalacia and a pulmonary edema syndrome in swine. *Appl. Environ. Microbiol.* **1990**, *56* (10), 3225−3226.

Shephard, G. S.; Sydenham, E. W.; Thiel, P. G.; Gelderblom, W. C. A. Quantitative determination of fumonisins B_1 and

B_2 by high-performance liquid chromatography with fluorescence detection. *J. Liq. Chromatogr*. **1990**, *13* (10), 2077−2087.

Sydenham, E. W.; Theil, P. G.; Marasas, W. F. O.; Shepard, G. S.; VanSchalkwyk, D. J.; Koch, K. R. Natural occurrence of some Fusarium mycotoxins in corn from low and high esophageal cancer prevalence areas of the Transkei, Southern Africa. *J. Agric. Food Chem*. **1990**, *38*, 1900−1903.

Thiel, P. G.; Marasas, W. F. O.; Sydenham, E. W.; Shepard, G. S.; Gelderblom, W. C. A.; Nieuwenhuis, J. J. Survey of fumonisin production by *Fusarium* species. *Appl. Environ. Microbiol*. **1991**, *57* (4), 1089−1093.

Wilson, T. M.; Ross, P. F.; Owens, D. L.; Rice, L. G.; Jenkens, S. J.; Nelson, H. A. Experimental production of ELEM. A study to determine the minimum toxic dose in ponies. *Mycopathologia* **1992**, *117*, 115−120.

Received for review January 14, 1998. Revised manuscript received April 10, 1998. Accepted April 16, 1998. This publication was partially supported by Cooperative Agreement FD-000431 from the FDA and the National Center for Food Safety and Technology.

JF9800312