Purification of Fumonisin B_2 Isolated from Rice Culture

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Procedures are presented for the production, isolation, and purification of the mycotoxin fumonisin B_2 from cultures of *Fusarium moniliforme* MRC 826 grown on rice. Fumonisins B_1, B_2, and B_3 were extracted with acetonitrile:water, and the extracts were filtered and reduced in volume with a rotary evaporator. Fumonisin B_2 was isolated from the other fumonisins B_1 and B_3 by preparative reversed-phase liquid chromatography (LC). Fractions that contained fumonisin B_2 were concentrated to remove acetonitrile, and the remaining water fraction was frozen and freeze dried. The freeze-dried material was dissolved in a minimum amount of methanol and separated by preparative, centrifugally accelerated, radial, thin-layer chromatography on a silica gel-coated plate. An applicator that precisely controlled the rate of application of extract to the sorbent material was used to minimize the width of the band of extract. Fractions were eluted from the spinning plate with a linear gradient of (A) chloroform:acetone (4:3) and (B) methanol:acetone (1:1) applied at a rate of 3 mL/min. Gradient starting conditions were 10% B and 90% A, and ending conditions were 50% A and B. Fractions that contained fumonisin B_2 were combined and freeze dried. Recovery of purified fumonisin B_2 was 90% of that of the starting extract, and purity as determined by HPLC light scattering detection was greater than 90%.

Keywords: *Fumonisin B_2, rice culture material, purification FB_2*

INTRODUCTION

Large amounts of the mycotoxins fumonisin B_1, fumonisin B_2, and fumonisin B_3 (Figure 1), isolated from *Fusarium moniliforme* Sheldon, are required for toxicity studies due to recent investigations that implicate fumonisin B_1 and fumonisin B_2 as possible factors in the etiology of esophageal cancer, which occurs at very high rates in the Transkei region of South Africa and in other isolated regions of the world (Rheeder et al., 1992; Marasas et al., 1988a; Gelderblom et al., 1988; Bezuidenhout et al., 1988). A fatal disease of horses, equine leucoencephalomalacia (ELEM), in which the white and gray matter of the brain develop liquefactive lesions, occurs after ingestion of either purified fumonisin B_1 or culture material that contained fumonisins (Ross et al., 1991, 1994; Kellerman et al., 1990; Plattner et al., 1990; Marasas et al., 1988b). Fumonisin B_1 and culture material containing fumonisins are toxic to broiler chicks, ducklings, and turkey poults (Javed et al., 1995; Hall et al., 1995; Dombrink-Kurtzman et al., 1993; Javed et al., 1993; Brydon et al., 1987). Porcine pulmonary edema (PPE) develops in swine dosed with fumonisin B_1 or fumonisin-containing culture material (Colvin and Harrison, 1992; Harrison et al., 1990; Kriek et al., 1981). Studies with rats fed diets of purified fumonisin B_1 showed that liver and kidney are target organs of the toxins (Voss et al., 1993). Chemical compounds that are structurally similar to the fumonisins have been found to produce toxicity in animals and plants. AAL toxin, one ester of 1,2,3-propanetricarboxylic acid esterified to the backbone of 1-amino-11,15-dimethylheptadeca-2,4,5,13,14-pentol, is structurally related to fumonisin B_1 and fumonisin B_2 (Bottini and Gilchrist, 1981; Bottini et al., 1981). AAL toxin is cytotoxic to mammalian liver cells (Shier et al., 1991), rat liver cells, and dog kidney cells (Mirocha et al., 1992).

The mode of action of fumonisins is believed to be inhibition for the conversion of sphinganine to *N*-acylsphinganines (Wang et al., 1991). This causes elevated levels of free sphinganine and depletion of complex lipid metabolism. The role that fumonisin plays in animal diseases, its possible effects on humans, and its unique effect on sphingolipid biochemistry have created a need for methods to obtain large quantities of highly purified mycotoxins for toxicology studies.

Cawood et al. (1991) developed a preparative procedure for obtaining fumonisin B_1 using Amberlite, XAD-2, silica gel, and C_{18} reversed-phase chromatography. Total recovery of fumonisin B_1 by their method was 80%, and purity was $\geq$90%; however, recovery values were not presented on the concentration of B_1 at 90% purity or above. Another procedure was reported for the separation and purification of B_1 using XAD-2 column chromatography and high-performance liquid chromatography (HPLC) equipped with a C_{18} reversed-phase column (Vesonder et al., 1990). The purity obtained was estimated to be 95% by gas chromatography/mass spectrometry (GC/MS). However, we were not able to find any published procedures on the separation and purification of fumonisins B_2 and B_3. In the present investigation, we describe a method for the isolation and purification of fumonisin B_2 by preparative liquid chromatography and preparative, centrifugally accelerated, radial, thin-layer chromatography.

MATERIALS AND METHODS

Rice Culture. Rice (Uncle Ben's Converted Rice, 300 g) and distilled water (300 mL) were placed in 2.8 L Fernbach flasks. The flasks were held for 12 h at room temperature,

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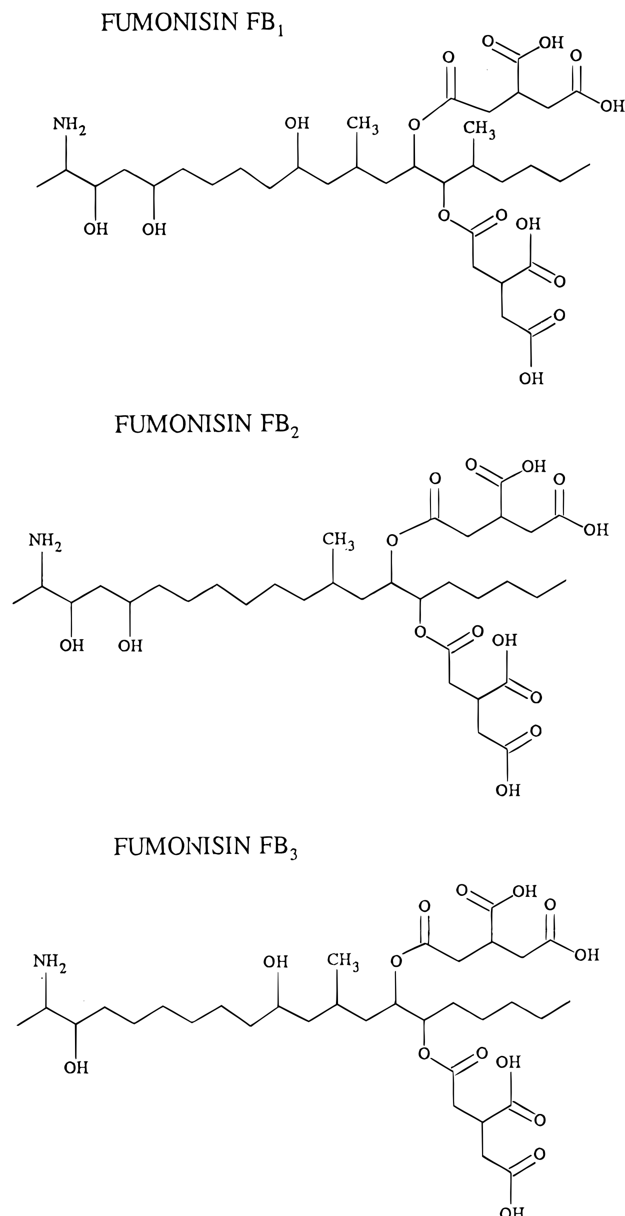

Figure 1. Chemical structures of fumonisins B_1, B_2, and B_3.

autoclaved for 30 min, and allowed to cool to room temperature. A 5 mL inoculum of an aqueous suspension of *F. moniliforme* MRC 826 conidia (10^9/mL) was added, and the flasks, stoppered loosely with a cotton plug, were incubated at 26 °C for 28−35 days in the dark. The flasks were shaken once daily for the first 10 days during the incubation period.

Extraction. Rice culture material (RCM) was extracted with water:acetonitrile (1:1) in the ratio of 100 g of RCM to 500 mL of solvent. The RCM was stirred occasionally during the extraction and after 4 h was vacuum filtered through Whatman #4 paper. The filtered RCM was resuspended in fresh water:acetonitrile (1:1) and allowed to stand overnight. The RCM was filtered as described above, and the extracts were combined. The extract volume was reduced approximately 60% using a 10 L vacuum rotary evaporator. The water bath temperature for all vacuum rotary evaporations was at 38 °C. This made the final acetonitrile concentration in the extract about 18−20%. The concentrated extract was placed into 4 L brown bottles and stored at 2 °C until preparative chromatography was carried out.

Analytical HPLC. Fumonisin B_1 and fumonisin B_2 were separated by HPLC and quantified by fluorescence detection after derivatization with *o*-phthalaldehyde (OPA) by a previously described procedure (Meredith et al., 1996).

Fumonisin B_2 (not derivatized) was also determined on a HPLC system equipped with an evaporative light-scattering detector (ELSD) (Alltech Associates, Deerfield, IL). This mass detector has a detection limit of 50 ng for fumonisin B_2. A Waters 6000 pump modified with glass pump heads (Bodman Industries, Aston, PA) was operated at 0.6 mL/min isocratically with the eluting solvent 75% methanol and 25% water. The solvent pH was adjusted to 2.43 by the addition of trifluoroacetic acid. Separation of the components was on a C_{18} reversed-phase Microsorb column, 10 cm × 4.6 mm, with 3 μm particle size (Rainin Instrument Co., Woburn, MA).

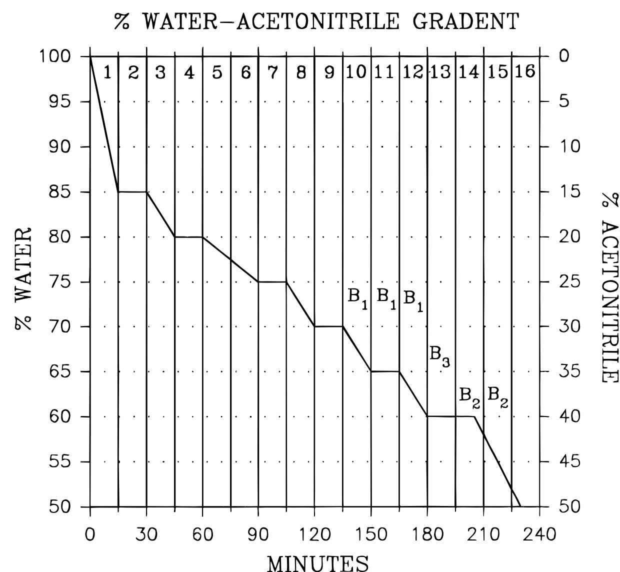

Figure 2. Water−acetonitrile gradient used during LC preparative separation of B_1, B_3, and B_2 and the 16 fractions collected.

Fumonisins B_1 and B_2 Standards. An analytical standard of fumonisin B_1 was prepared by the method of Plattner and Brenham (1994). The fumonisin B_2 standard was obtained from Sigma Chemical Co. (St. Louis, MO). Comparison by fluorescent HPLC and HPLC using ELSD of B_2 from Sigma Chemical Co. and a B_2 high-purity standard showed values similar in percent area counts (93% fluorescent detector) and (94.5% ELSD). Cochromatography of the two B_2 standards showed coelution with only one peak being eluted.

Preparative LC Separation of Fumonisins B_1 and B_2. The preparative LC procedures for partially separating fumonisin B_1 from fumonisin B_2 and B_3 using a water−methanol gradient and C_{18} reversed-phase cartridge were previously described (Meredith et al., 1996). The present procedure consisted of a water−acetonitrile step gradient used for elution in which the starting condition was water 100%, and after 230 min the ending condition was 50% water and 50% acetonitrile (Figure 2). This solvent system completely separated fumonisins B_1, B_2, and B_3.

Fractions were collected and analyzed by fluorescence HPLC for the OPA derivative of fumonisins B_1, B_2, and B_3. Rotary evaporation was used to remove the acetonitrile from the two fractions containing fumonisin B_2. The fractions were combined, frozen, and freeze dried. The partially purified fumonisin B_2 material was stored at −27 °C until additional purification could be conducted.

Purification of Fumonisin B_2. A Chromatotron (Harrison Scientific, Palo Alto, CA) was used to purify fumonisin B_2. A round TLC plate coated with 4 mm thick TLC grade silica gel (Merck 7749 with gypsum binder) was dried under a 100 W light bulb, and the dry sorbent was scraped to 2 mm thickness. The sorbent was activated by heating at 70 °C for 12 h.

The partially purified fumonisin B_2 sample was redissolved in a small amount of methanol and was applied to the spinning TLC plate using a holder that held a 10 mL syringe attached to the Chromatotron with teflon tubing. The TLC plate was operated under 1 atm of nitrogen. The syringe holder (Figure 3) was equipped with a rotary screw handle used to depress the syringe plunger to control the rate of sample applied. This

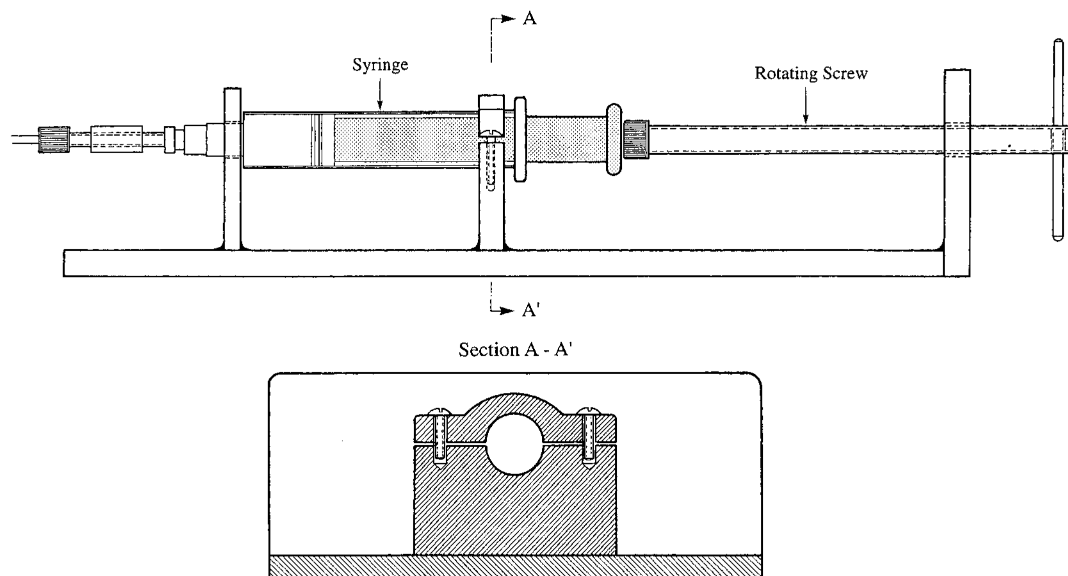

Figure 3. Syringe holder with screw for precisely controlling the quantity of liquid applied to the centrifugal TLC plate.

syringe holder allowed the application of sample to the sorbent at a very slow rate, resulting in a very small colored band of material that was less than 5 mm wide.

Various solvent systems were studied to determine the best system to isolate fumonisin B_2 from other compounds on the silica gel TLC plate. Two Altex (Beckman, Fullerton, CA) 110 pumps, Altex 420 gradient controller, and Altex solvent mixer were used to supply solvent to the spinning silica gel TLC plate. The solvent consisted of (A) chloroform−acetone (4:3) and (B) methanol−acetone (1:1) applied as a linear gradient over 180 min at 3 mL/min. The starting solvent gradient was 90% (A) and 10% (B), and the ending gradient was 50% (A) and 50% (B). Fractions (6 mL) were collected with a fraction collector and dried on a Savant Speedvac (Farmingdale, NY). The fractions were redissolved in 2 mL of acetonitrile-water (1:1), and every fifth fraction was analyzed by HPLC (fluorescent detection) to determine which fractions contained B_2. Fractions containing fumonisin B_2 were also analyzed by HPLC using ELSD as a detector. The fractions containing fumonisin B_2 were combined and placed on a rotary evaporator, and the acetonitrile was removed. The remaining water was frozen, and the samples were freeze dried to obtain the purified B_2.

RESULTS AND DISCUSSION

Rice as a substrate for *F. moniliforme* MRC 826 produced about the same quantity of fumonisin B_1 and B_2 as corn (Meredith et al., 1996) and gave cleaner extracts with less small fines than cultures grown on corn. This reduced problems with check valves on the preparative pumps. The extract also filtered faster and had less colored pigments than extracts prepared from cultures grown on corn. We also have observed that extracts from rice are easier to purify and that purity is higher than extracts from corn.

Sixteen fractions were collected in 230 min from the C_{18} reversed-phase preparative cartridge using a starting gradient of 100% water and ending with 50% water−acetonitrile (Figure 2). The use of acetonitrile instead of methanol in the gradient eliminates the possibility of methylation of the compounds of interest. Fumonisins B_1, B_3, and B_2 were completely separated from each other by this solvent system. Fractions 14 and 15 contained partially purified fumonisin B_2 with a total recovery of 974 mg (from 900 g of RCM) from the combined fractions.

The first 18 fractions (108 mL, total volume) that eluted from the centrifugal TLC plate were red in color. All the remaining fractions that were collected were colorless. TLC fraction 22 was the first fraction to contain fumonisin B_2. The concentration of fumonisin B_2 increased in each fraction until fraction 25 and then remained about the same until fraction 45. After fraction 45, fumonisin B_2 levels decreased.

The original extract, which was dark red, and the TLC fraction 35 were analyzed by HPLC (Figure 4A,B) using fluorescent detection of the OPA-derivatized fumonisins. The chromatogram of the original extract (Figure 4A) shows that fumonisin B_2 is the major fluorescent component (56% based on area counts) and that fluorescent contaminates were greatly reduced by the centrifugal TLC purification process (greater then 90% based on area counts) (Figure 4B). The chromatogram of fraction 35 is representative of the fractions from TLC fractions 25−45. The red pigments in the original extract were eluted in the first 18 fractions of the centrifugal TLC run, and the eluted fractions containing fumonisin B_2 contained no pigments as they were colorless. The HPLC chromatogram using ELSD of the TLC fraction 35 shows that fumonisin B_2 is the major constituent (94% area counts) of this fraction (Figure 5).

The amount of fumonisin B_2 applied to the centrifugal TLC plate was approximately 974 mg, and the recovered fumonisin B_2 from all combined TLC fractions was 864 mg, which represents a recovery of better than 90%. The centrifugal spinning TLC system should be able to be scaled up so that gram quantities of material can be applied with the use of thicker silica gel absorbent (4 mm) on the plates. The purity (90% or greater) of fumonisin B_2 was greatly improved using this method over the procedure of Cawood et al. (1991). Rechromatography of purified fumonisin B_2 on the centrifugal TLC system with slight differences in the solvent system or in the activation of the silica gel may increase the purity. The procedures described are rapid and require small quantities of solvent compared to the method of Cawood et al. (1991), which require large chromatographic columns and liters of solvent for the purification of fumonisin B_2.

SUMMARY

Inoculation with *F. moniliforme* on rice and corn produced similar quantities of fumonisins B_1 and B_2.

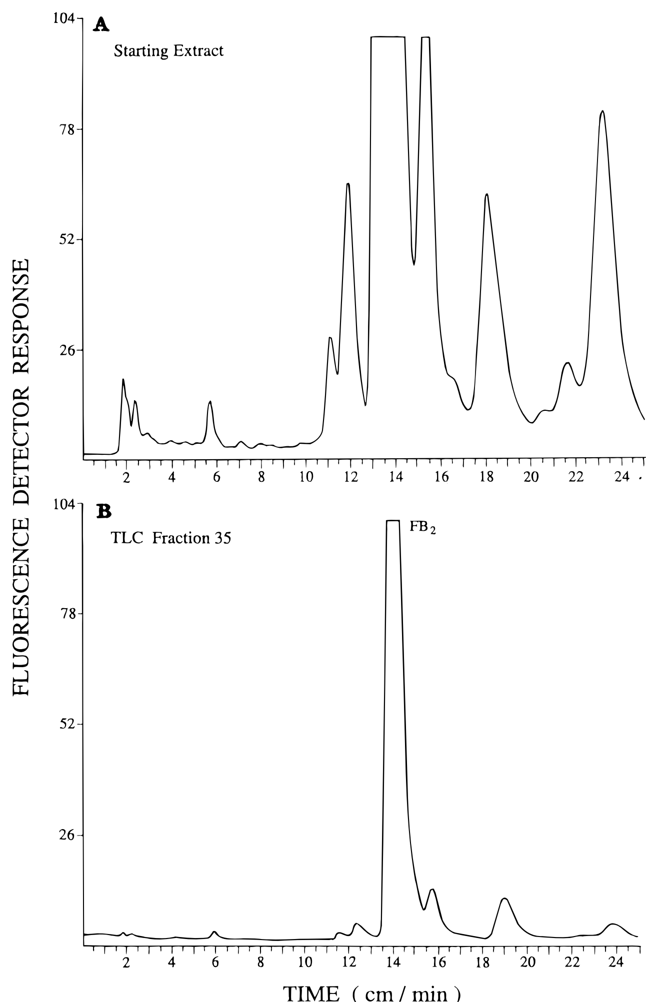

Figure 4. (A) Extract from LC preparative separation that contained B_2 and contaminates. (B) TLC Fraction after purification by preparative, centrifugally accelerated, radial thin-layer chromatography. Detection was by HPLC/fluorescence detector.

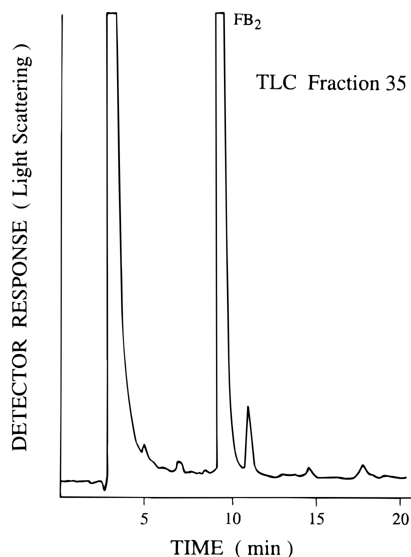

Figure 5. Determination of B_2 in TLC fraction 35 by HPLC/ELSD after preparative, centrifugally accelerated, radial thin-layer chromatography.

The RCM produce cleaner extracts than corn and reduced the problems with check valves in the preparative LC systems. Pigments produced with rice were less than with corn. Thus, the extracts from rice were easier to purify. The use of an acetonitrile–water gradient rather than a methanol–water gradient resulted in improved separation of fumonisins B_1, B_2, and B_3, reduced the amount of organic solvent required, shortened chromatography time, and eliminated the chance of methylation of fumonisin B_1 and fumonisin B_2 by exposure to methanol. Centrifugal TLC purification of fumonisin B_2 gave good results as fumonisin B_2 was obtained in good quantity and high purity (better then 90%). The method is fast and requires small amounts of solvents.

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