Purification of Fumonisin B_1 from Liquid Cultures of *Fusarium proliferatum*

Keywords: *Liquid fermentation; corn hull extract; fumonisin; FB_1; Fusarium proliferatum*

INTRODUCTION

Fumonisins (FBs) are a family of mycotoxins (FB_1, FB_2, FB_3, and FB_4) that are produced by the maize pathogens *Fusarium proliferatum* and *Fusarium moniliforme*. Purification of FBs has been pursued for the purpose of establishing their acceptable levels in foods for human and animal consumption. The FBs have been purified from solid corn cultures of *F. moniliforme* strain MRC 826. Gelderblom et al. (1988) and Cawood et al. (1991) purified FBs by extraction, solvent partitioning, XAD (adsorption)-2, silica gel (adsorption), and C_{18} (partition) liquid chromatography (LC). Cawood et al. (1991) obtained ≥90% pure FB_1 with a 40% recovery. Voss et al. (1993) obtained ≥99% pure FB_1 by XAD-2 and repeated C_{18} LC steps. Miller et al. (1994) obtained 97% pure FB_1 (89% recovery) produced from liquid cultures of *F. moniliforme* strain MRC 826 by DEAE-Sephadex (ion exchange), silica gel, and gradient C_{18} LC. Recently, Meredith et al. (1996) obtained >95% pure FB_1 with a >90% recovery from solid rice cultures of *F. moniliforme* strain MRC 826 using C_{18} and cyano (partition) LC. In our laboratory, we have obtained only 85% pure FB_1 from solid corn or rice cultures of *F. proliferatum* strain M5991 by XAD-16, C_8 (partition), silica gel, and gradient C_{18} LC. Using liquid cultures of *F. proliferatum* strain M5991, we have achieved a reproducible purification method of obtaining >95% pure FB_1 with a 37% recovery using XAD-16, C_8, DEAE-Sepharose (ion exchange), and C_{18} LC.

MATERIALS AND METHODS

The FB_1 produced from *Fusarium* cultures is a class 2B carcinogen (IARC, 1993) and caution should be taken during its handling.

Liquid Cultures. Cultures of *F. proliferatum* strain M5991 were grown in liquid modified Myro medium containing ≤1.00% (v/v) corn hull extract (CHE) (Dantzer et al., 1996). Modified Myro medium consisted of 1.0 g of $(NH_4)_2HPO_4$, 3.0 g of KH_2PO_4, 0.5 g of $MgSO_4{\cdot}7H_2O$, 5.0 g of NaCl, 40 g of sucrose, and 10 g of glycerin in 1 L of distilled water (dH_2O), pH 5.9. The CHE was prepared by autoclaving 10 g of corn hulls with 100 mL of dH_2O for 1 h at 121 °C followed by centrifugation at 13776*g* for 10 min. The clarified CHE was made to 100 mL volume using dH_2O, sterilized at 121 °C for 20 min, and stored at ambient temperature until needed. The cultures produced ≤750 mg of crude FB_1/L. Upon harvest, the liquid cultures were centrifuged and passed through a Whatman No. 1 filter (Whatman Laboratory Division, Springfield Mill, Maidstone, Kent) followed by 0.8 and 0.45 μm MSi filters (4.5 cm diameter, nylon; Micron Separations Inc., Westboro, MA).

XAD-16 Adsorption LC. A gravity-fed LC column was made with Amberlite nonionic polymeric adsorbent XAD-16 (surface area, 800 m^2/g; average pore diameter, 100 Å; wet mesh size, 20−60; Sigma Chemical Co., St. Louis, MO) by transferring 1 kg of dry XAD-16 material into a 100 × 7.5 cm glass column containing a fritted glass filter. The XAD-16 was washed with >2 L of 50% acetonitrile (ACN), until the eluent was clear, followed by 6 L of dH_2O, and allowed to drain to the top of the column bed. Filtered liquid cultures containing ≤2 g of FB_1 were introduced onto the column. The XAD column was washed with 4 L of dH_2O and drained completely. The FBs were eluted with 4 L of 50% ACN. The XAD column was regenerated with 4 L of dH_2O. Eluted FB-containing fractions were concentrated by rotary evaporation (RE) to 30 mL (Rotavapor, Buchi/Brinkman Instruments Inc., Westbury, NY) at 50 °C and filtered through a 0.45 μm MSi filter. The FB-containing filtrates were adjusted to a pH ≤3.3 using 6 M hydrochloric acid (HCl).

C_8 Partition LC. The C_8 mobile phase solutions contained 0.1% (v/v) trifluoroacetic acid. The column pressure was maintained at 80 psi throughout. The low-pressure C_8 LC column (Lobar C8, LiChroprep RP-8, 40−63 μm, size B; 310-25; EM Separations, Gibbstown, NJ) was conditioned by pumping 500 mL each of ACN and dH_2O through the column with a metering pump (LDC analytical minipump Model 396, Rainin Instruments Co. Inc., Woburn, MA). The pH-adjusted filtrates were partitioned to contain ≤0.5 g of FB_1 and introduced onto the column via one to three injections (10 mL of filtrate per injection) with a Rheodyne injection valve (Model 7010, Rheodyne Inc., Cotati, CA) and an inline 10 mL injection loop. The column was washed with 250 mL each of dH_2O and 20% ACN followed by 150 mL of 25% ACN. The FB_1 was eluted with 400 mL of 30% ACN into 12.5 × 1.5 cm tubes (10 mL of eluent/tube) using a fraction collector (FRAC-100, Pharmacia Fine Chemicals, Piscataway, NJ). The eluents were monitored for FB_1 by thin layer chromatography (TLC), concentrated by RE to 10 mL, and filtered through a 0.45 μm MSi filter, and the pH was adjusted to 6.5 using 10 N NaOH. The remaining FBs and other contaminants were eluted from the column with 100 mL of ACN. The column was regenerated with ≥250 mL of dH_2O.

DEAE-Sepharose Ion Exchange LC. All of the ammonium acetate (NH_4-Oac) solutions were pH adjusted to 6.5 using 10 N NaOH. Initially, DEAE-Sepharose (65 mL) CL-6B (wet bead size, 45−165 μm, Sigma) was washed with 650 mL of Milli-Q H_2O (MqH_2O), resuspended in 1.0 M NH_4-OAc:ACN [1:1], and stored at 5 °C for 2 days with several resuspensions per day. The DEAE-Sepharose was washed with MqH_2O until no NH_4-OAc odor remained, degassed in 50% ACN, transferred into a 30 × 2.5 cm column, and allowed to settle by gravity. The column was washed with 200 mL of 50% ACN and drained to the top of the bed.

The FB_1-containing fractions were partitioned to contain ≤0.5 g of FB_1 and transferred to the DEAE-Sepharose column. The sample was allowed to filter onto the column to the top of the bed followed by 10 mL of 50% ACN, which was allowed to drain to the top of the bed. The column was washed with 90 mL of 50% ACN followed by 100 mL each of 100 and 200 mM NH_4-OAc:ACN [1:1]. The FB_1 was eluted with 100 mL each of 300 and 400 mM NH_4-OAc:ACN [1:1]. The DEAE-Sepharose were regenerated with 100 mL of 500 mM NH_4-OAc:ACN [1:1] followed by 200 mL of 50% ACN. The eluents were monitored for FB_1 by TLC, concentrated by RE to 10 mL, and filtered through a 0.45 μm MSi filter, and the pH was adjusted to ≤3.3 using 6 N HCl.

C_{18} Partition LC. The C_{18} LC mobile phase solution contained 0.1% acetic acid and was pumped at a flow rate of 2 mL/min throughout. The YMC-Pack semipreparatory C_{18} high-performance liquid chromatography (HPLC) column (AM 323-5, size, 25 × 1 cm i.d.; particle, S-5 μm, 120A; YMC Inc., Wilmington, NC) was washed with 60 mL of ACN followed by 60 mL of MqH_2O. After MqH_2O equilibration, the FB_1-containing fractions were loaded onto the column by two injections (5 mL each) with a Rheodyne injection valve (Model 7010) and an in-line 5 mL injection loop. The column was washed with 60 mL each of 0, 20, and 25% ACN, respectively. The FB_1 was eluted with 60 mL each of 30 and 35% ACN into 12.5 × 1.5 cm tubes (10 mL of eluent/tube) using a fraction collector (Pharmacia Fine Chemicals). The collected eluents were monitored for FB_1 by TLC. Eluents containing FB_1 were

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Table 1. Fumonisin B_1 (FB_1) Mass, Recovery, and Purity Obtained from Liquid Cultures of *F. proliferatum* Strain M5991 by XAD-16, C_8, DEAE-Sepharose, and C_{18} Liquid Chromatography[a]

culture sample	CHE (%)	crude FB_1 (mg)	pure FB_1 (mg)	FB_1 recovery (%)	FB_1 purity (%)
61	0.00	400	267	67	101
62	0.00	640	169	27	98
64	0.00	522	287	55	109
75	1.00	600	379	63	101
83	0.75	600	151	25	98
84	0.50	280	82	30	102
106	1.00	750	126	17	101
117	0.00	87.5	57	66	100
120	1.00	480	92	19	96
total		4360	1612	37	102

[a] Liquid cultures were incubated in Myro medium containing 0–1.00% (v/v) corn hull extract (CHE) for >50 days at 22.5 ± 2.5 °C.

concentrated by RE to 10 mL at 50 °C and filtered through a 0.45 μm MSi filter. The column was reconditioned by washing with 60 mL each of 40, 100, and 0% ACN, respectively.

TLC. All LC fractions were evaluated for FBs by TLC (Rottinghouse et al., 1992). Fractions containing FB_1 were identified by observing the fluorescence of the FB_1–fluorescamine compound under UV light.

Freeze-Drying. Filtered FB_1-containing eluents were concentrated by RE, resuspended in 10–15 mL of MqH_2O, and lyophilized in a freeze-dryer (18 port, Labconco Corp., Kansas City, MO) for >12 h.

Fumonisin Analysis. Concentrations of FB_1 were estimated by HPLC *o*-pthaldialdehyde (OPA) fluorometry (Hopmans and Murphy, 1993). Purity of FB_1 was determined by comparing the freeze-dried weight of a purified FB_1 culture sample to that of the HPLC-OPA quantified amount. The FB_1 standard curve was prepared with FB_1 generously donated by P. G. Thiel (Research Institute for Nutritional Diseases, South African Medical Research Council, South Africa). For independent confirmation of purity, sample 84 was analyzed using HPLC-OPA fluorometry and electrospray mass spectrometry (MS) by Dr. Ronald Plattner (National Center for Agricultural Utilization Research, Peoria, IL).

RESULTS AND DISCUSSION

The results of nine representative liquid cultures are presented in Table 1. An average FB_1 purity of 102% with a 37% recovery was obtained. There were no differences in FB_1 purity or recovery from the liquid cultures containing different levels of CHE. A purification flow chart is presented in Figure 1. The crude FB_1 in the liquid cultures had a purity of 7%. The post-XAD-16 FB_1 purity was ≥50%. During the C_8 LC, FB_1 was eluted with 30% ACN; however, we observed FB_1 elution during the 25% ACN wash. Therefore, we decreased the volume of this wash from 250 to 150 mL. We limited the loading of FB_1 onto our XAD-16 and C_8 columns to 2 and 0.5 g, respectively. These mass limits of FB_1 did not cause overloading of the columns by FBs or contaminants. Previously, we obtained ≥95% pure FB_1 by repeated DEAE-Sepharose and C_{18} LC. The average recovery of FB_1 (37%) in Table 1 was similar to the 40% recovery obtained by Cawood et al. (1991). Figure 2 presents chromatograms of sample 84 containing ≥95% pure FB_1 (A) and a water blank (B) analyzed by HPLC-OPA fluorescence. This figure shows the lack of any OPA-derivatizable contaminants in this sample purified according to our procedure. This same sample was analyzed by Dr. Plattner using HPLC-OPA fluorometry (95.9% FB_1) and by electrospray-MS (96.8% FB_1) (Figure 3).

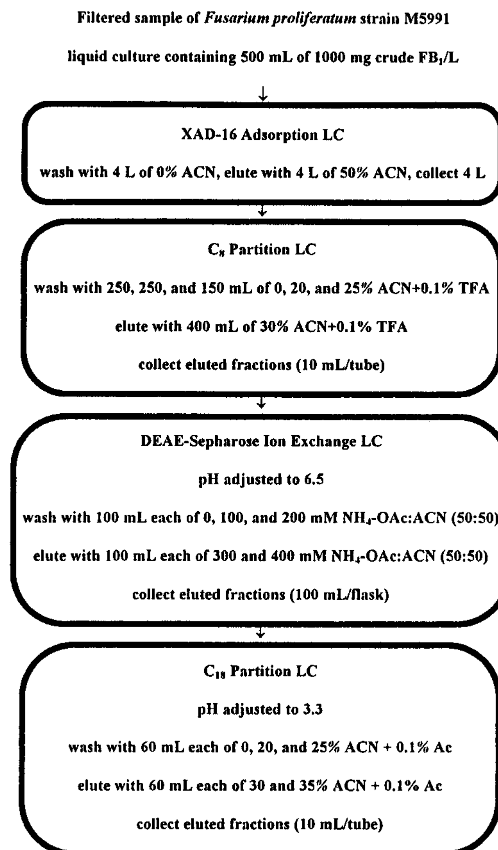

Figure 1. Purification procedure for fumonisin B_1 (FB_1).

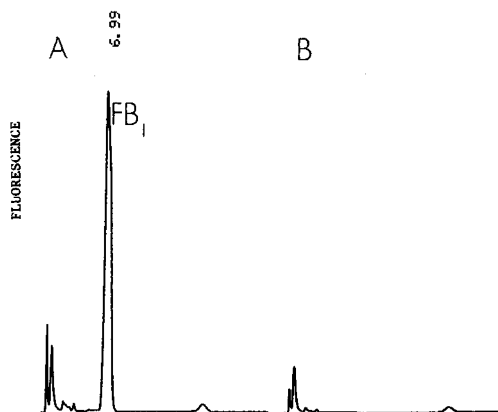

Figure 2. HPLC-OPA chromatogram of sample 84 (4.6 mg of fumonisin B_1/L) (A) and a water blank (B).

We were unable to obtain pure FB_1 from solid corn, solid rice, or liquid Myro media using Miller's (Miller et al., 1994) silica or Meredith's (Meredith et al., 1996) cyano LC procedures. Our group found that FB_1 produced by *F. proliferatum* strain M5991 from liquid cultures was easier to purify than FB_1 produced on solid corn medium. The principal FB produced by *F. proliferatum* strain M5991 is FB_1 (>90%). We believe that FB_1 purified from liquid cultures of this strain has an advantage over that of other FB producers because of the production of high levels of FB_1 and low levels of other FBs.

We have demonstrated an alternate and reproducible method for purification of FB_1 from liquid cultures of *F. proliferatum* strain M5991. This method could be applied to the purification of FB_2, FB_3, and FB_4 (work in progress) since they can be separated from FB_1 during C_8 LC. In addition, purification and recovery of

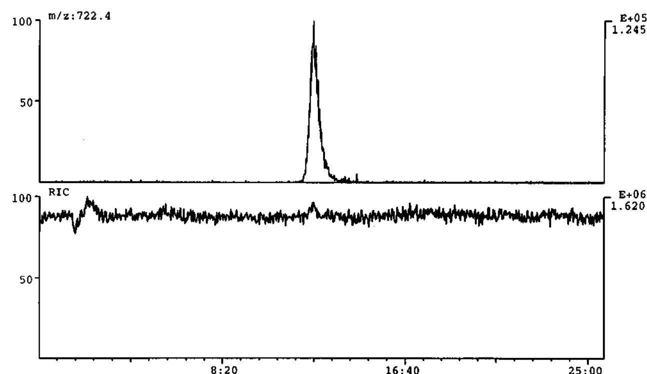

Figure 3. Chromatogram of underivatized fumonisin B_1 in sample 84 analyzed by Dr. R. Plattner using electrospray interface-MS detection (mode, ESI +Q1MS LMR UP LR; peak, 1000.00 mmu; masses scanned, *m/z* 250−950).

FB_1 were not altered by the addition of CHE to the liquid Myro medium.

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