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Production, purification, 14C labeling, and bioavailability of fumonisin B1 produced by Fusarium proliferatum strain M-5991

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Production, purification, ^{14}C labeling, and bioavailability of fumonisin B$_1$ produced by

Fusarium proliferatum strain M-5991

by

William Raymond Dantzer

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Co-Majors: Food Science and Technology; Toxicology

Major Professors: Patricia A. Murphy and Suzanne Hendrich

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For the Major Program

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For the Graduate College

Dedication

I am pleased to dedicate this dissertation to my beloved wife. Daniele. who constantly fed me encouragement and supported me through my ups and downs during this work. I am also pleased to dedicate this to by my parents, Blanche and Albert, to whom I am deeply indebted.

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ABSTRACT

The production of FB_1 by *Fusarium proliferatum* M-5991 was enhanced from 300 to 1400 µmoles/L by the addition of 0.75% corn hull extract (CHE) to modified Myro liquid medium (LM). Three continuous batch fermentations of *F. proliferatum* M-5991 in 10. 12. and 12 L modified Myro LM produced 28 mmoles of FB_1 which were purified to >95% by a combination of XAD-16, C_8, DEAE-Sepharose, and C_{18} chromatography. At day 12 of culture, $[^{14}C]$-acetate was incorporated into 29 µmoles of FB_1 in 50 ml modified Myro LM containing *F. proliferatum* which produced, after purification, 24 µmoles $[^{14}C]$-FB_1 at 95% purity with a specific activity of 1.7 mCi/mmole. In a urinary excretion study, 9 male and 9 female Fisher F344/N rats were gavaged with 0.69 µmoles of $[^{14}C]$-FB_1 or $[^{14}C]$-hydrolyzed FB_1 ($[^{14}C]$-HFB_1) or $[^{14}C]$-FB_1-FRU ($[^{14}C]$-FB_1-FRU)/kg body weight (BW). The urinary excretion by male and female rats was 12.8 and 17.3% of the $[^{14}C]$-HFB_1 dose, 4.2 and 4.6% of the $[^{14}C]$-FB_1-FRU dose, and 0.4 and 0.7% of the $[^{14}C]$-FB_1 dose, respectively. In a biliary excretion study, 9 Sprague-Dawley female rats were dosed by gavage with 0.69 µmole $[^{14}C]$-FB_1 or $[^{14}C]$-HFB_1 or $[^{14}C]$-FB_1-FRU/kg BW. There was no difference in the biliary excretion of the 3 FB compounds by these rats with 1.4% of the dose being excreted in the bile 4-h after dosing. These results, that HFB_1 was absorbed to a greater extent by rats than FB_1, complement previous reports that FB_1 toxicity was $\geq HFB_1$ toxicity in cell cultures and as toxic on a dietary basis in rats. Because dietary FB_1-FRU was absorbed to a greater extent than FB_1, absorption differences between the two compounds do not explain the lack of toxicity of FB_1-FRU in rats.

CHAPTER 1. GENERAL INTRODUCTION

A. Introduction

The purpose of this dissertation is to describe the production, purification, and radiolabeling of fumonisin B_1 (FB_1), and evaluate the urinary and biliary excretion of [^{14}C]-FB_1, [^{14}C]-hydrolyzed FB_1 (HFB_1), and [^{14}C]-FB_1-Fructose (FRU) by rats. The urinary and biliary excretion of these compounds were studied to gain insight into their bioavailability in animals.

Fusarium moniliforme and *Fusarium proliferatum* have been shown to produce FB_1 and other FBs, along with other mycotoxins, in field corn or in defined mediums such as solid corn medium (SCM), solid rice medium (SRM), or liquid medium (LM) (Gelderblom *et al.*, 1988; Cawood *et al.*, 1991). These fungal pathogens invade maize plants by many pathways including insect damaged kernels and may result in maize diseases such as stalk and ear rot and kernel infection (Ross *et al.*, 1991; Nelson, 1992).

B. Dissertation Organization

This dissertation is organized into 6 chapters. Chapter 1 is a general introduction; Chapter 2 is a literature review; Chapters 3 and 4 are published work on the production and purification of FB_1 (Dantzer *et al.*, 1996a and 1996b); and Chapter 5 is a manuscript to be submitted for publication on the bioavailability of 0.69 μmoles of [^{14}C]-FB_1 or [^{14}C]-HFB_1 or [^{14}C]-FB_1-FRU/kg BW, as determined by urinary and biliary excretion, by rats. All figures and tables are renumerated for each Chapter or Appendix.

Because Chapters 3 and 4 are already published, there were some inconsistencies with abbreviations which were later found to be more appropriate. Following is a list of abbreviations and the chapters in which they are referenced. [Solid corn medium (SCM)-Chapter 2], [corn culture material (CCM)-Chapters 3 and 4]; [modified Myro liquid medium (modified Myro LM)-Chapters 2 and 5], [modified Myro medium (MM medium)-Chapters 3 and 4]; [μmole FB/L-Chapters 2, 5, and 6], [mg FB/L-Chapters 3 and 4]; [ml-Chapters 2 and 5], [mL-Chapters 3 and 4].

C. References

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CHAPTER 2. LITERATURE REVIEW

A. Why Study Fumonisins?

The fumonisin B series (FBs) refer to a group of mycotoxins (FB_1, FB_2, FB_3, and FB_4) that have a common 12,16-dimethyl-14,15-diesterpropane-1,2,3-tricarboxylate-hydroxyicosane structure (Fig. 1) and are produced mainly by *Fusarium moniliforme* and *Fusarium proliferatum* (Gelderblom *et al.*, 1988; Cawood *et al.*, 1991, Wilkes *et al.*, 1995; Beier *et al.*, 1995). The FBs are known to cause different diseases in horses, pigs, or rats (Marasas *et al.*, 1984). Fumonisin B_1 has been associated epidemiologically with human esophageal cancer (Sydenham *et al.*, 1990; Rheeder *et al.*, 1992; Chu and Li, 1994.). The fumonisin A series (FAs) and the fumonisin P series (FPs), also produced by *F. moniliforme* and *F. proliferatum*, are N-acetyl- and N-3-hydroxypyridinium-FB mycotoxins, respectively (Fig. 1) (Musser and Plattner, 1997).

Cultures of *F. moniliforme* Medical Research Council (MRC) in Tygerberg South Africa have been shown to produce FBs (Gelderblom *et al.*, 1988; Gelderblom *et al.*, 1991; Norred *et al.*, 1992; Voss *et al.*, 1993). Kriek *et al.* (1981) reported that *F. moniliforme* MRC-826 grown in solid corn medium (SCM) induced toxicities when fed to horses, pigs, sheep, rats, and baboons. Marasas *et al.* (1984) reported an induction of hepatic lesions and mortality in rats fed *F. moniliforme* MRC-826 grown in SCM. Jaskiewicz *et al.* (1987) reported an induction of liver cancer in rats fed *F. moniliforme* MRC-826 grown in SCM. However, these SCM may have contained many mycotoxins.

Equine leucoencephalomalacia (ELEM) is a brain disease which has been associated

with the consumption of FB-contaminated corn. Symptoms were induced in horses fed 14

μmoles of purified FB_1/kg diet (Marasas *et al.*, 1988). Symptoms of ELEM included

deterioration and liquefaction of the white-gray matter in the brain of a horse resulting in

ataxia, clumsiness, stupor, and death (Kellerman *et al.*, 1991). Porcine pulmonary edema

(PPE) is a lung disease that has been associated with the consumption of FB-contaminated

corn. Symptoms were induced in swine fed 35 μmoles of purified FB_1/kg diet (Harrison *et

al.*, 1990). Symptoms of PPE include leakage of serum through the lungs, filling the thoracic

cavities, and death. The FBs are not toxic to cattle (Osweiler *et al.*, 1993; Smith and Thakur,

1996) nor have they previously been reported in the milk of lactating cows fed corn

containing FBs (Richard *et al.*, 1996). Laboratory rats developed liver cancer and kidney

toxicity when fed 69 μmoles of purified FBs (Gelderblom *et al.*, 1991; Voss *et al.*, 1993).

The cancer causing ability of FB_1 is thought to be primarily due to its accumulatory effects

on sphingoid bases (Schroeder *et al.*, 1994). The FBs resemble sphinganine and sphingosine

and inhibit ceramide synthase, one of the enzymes involved in the conversion of sphinganine

to sphingosine (Merrill *et al.*, 1993). Mechanisms of FB toxicities may include interfering

with sphingolipid synthesis, inhibition of Na/K ATPase, inhibition of protein kinase C

(PKC), release of intracellular Ca^{2+}, promotion of RB dephosphorylation, and induction of

apoptosis (Merrill *et al.*, 1996). Mechanisms of FB carcinogenicities may include activation

of epidermal growth factors (EGF) receptor/MAP kinase, activation of phospholipase D

and/or inhibition of phosphatidic acid phosphohydrolase, release of intracellular Ca^{2-},

activation of AP-1, cytotoxicity for normal cells, loss of regulation of differentiation, loss of

regulation of apoptosis, and loss of lipid mediators of tumor necrosis factors caused by sphingolipid disruption (Merrill *et al.*, 1996).

The FBs are produced by fungi associated with corn. However, corn destined for animal foods has higher populations of these fungi suggesting a higher risk of FB contamination in such corn than in corn meant for human foods (Ross *et al.*, 1991). Fumonisins have been detected in corn products destined for humans (Hopmans and Murphy, 1993; Pohland, 1996; Bullerman, 1996). The effects on humans of consumption of FBs are not known. High incidences of esophageal cancer in humans have been associated with the consumption of FB containing corn (Chu and Li, 1994).

Because FBs have the ability to cause diseases in animals and their effects on humans are uncertain, it is necessary to take precautions to minimize the occurrence of FBs in foods. Measuring the bioavailability of FBs in laboratory animals can help us to understand the toxicokinetics of FBs and aid in establishing safe levels of FBs that may be consumed by animals and humans.

Processing of foods containing FB_1 can produce hydrolyzed FB_1 (HFB_1) in which the tricarboxylic acid (TCA) groups are removed from the FB_1 backbone (Fig. 1). Nixtamalization, the process for making tortilla flour or masa in which corn is subjected to alkaline heating and steeping, can form HFB_1 (Hendrich *et al.*, 1993; Murphy *et al.*, 1996). Another form of FB_1, FB_1-fructose (FB_1-FRU) was formed when FB_1 was heated in the presence of a reducing sugar at pH > 7 (Fig. 1). The amine group on FB_1 can condense with the carboxyl group on a reducing sugar such as glucose, fructose, or lactose, blocking the amine group and making the FB_1 molecule undetectable by using conventional derivatization

detection means. These modifications to FB_1 may alter its detection, bioavailability, and/or

toxicity (Bordson *et al.*, 1995; Jackson *et al.*, 1996a; Jackson *et al.*, 1996b; Murphy *et al.*,

1996). For the assurance of a safe human and animal food supply, all forms of FBs that may

be found in food products must be evaluated for their bioavailability, safety, and toxicity in

animals.
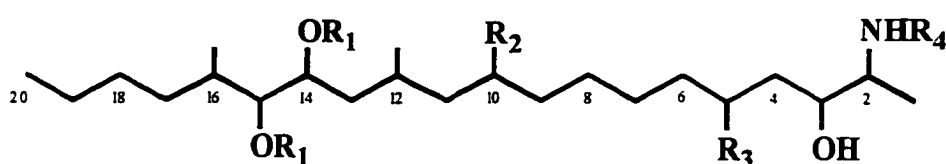
FUMONISIN	R_1	R_2	R_3	R_4
B_1	TCA	OH	OH	H
B_2	TCA	H	OH	H
B_3	TCA	OH	H	H
B_4	TCA	H	H	H
HFB_1	H	OH	OH	H
FB_1-FRU*	TCA	OH	OH	FRUCTOSE
A_1	TCA	OH	OH	$COCH_3$
A_2	TCA	H	OH	$COCH_3$
P_1	TCA	OH	OH	3HP
P_2	TCA	H	OH	3HP

Figure 1. Chemical structures of the major mycotoxins produced by *Fusarium moniliforme* and *Fusarium proliferatum*: fumonisin B series (FB_1, FB_2, FB_3, and FB_4), hydrolyzed fumonisin B_1 (HFB_1), fumonisin B_1-fructose conjugate (FB_1-FRU) (*may be formed during food processing), fumonisin A series (FA_1, FA_2), and fumonisin P series (FP_1 and FP_2). TCA = propane-1,2,3-tricarboxylic acid group [$COCH_2CH(CO_2H)CH_2CO_2H$]. 3HP = 3-Hydroxypyridinium [NC_5H_5O].

B. Fumonisins in Corn

1. *Fusarium* strains and toxins

The fungal species *F. moniliforme* Sheldon and *F. proliferatum* (Matsushima)

Nirenberg are in the *Liseola* section of the genus *Fusarium*. These species do not form

chlamydospores, contain unbranched and branched monophialide and/or polyphialide

macroconidiophores, and are distinguished by morphology, mode of microconidia formation.

and microconidiophore morphology (Nelson *et al.*, 1983; Nelson, 1992). The reproductive

state (perfect state) of *F. moniliforme* is called *Gibberella fujikuroi* (Sawada) Wollenw and

has been known to produce mycotoxins including deoxynivalenol, diacetoxyscirpenol.

fusaric acid, fusarins, gibberellins, moniliformin, T-2 toxin, zearalenone, and the fumonisins

(Marasas *et al.*, 1984; Nelson, 1992). Different fungal species normally are not classified as

having the same perfect state. However, the perfect state of *F. proliferatum* is also

Gibberella fujikuroi (Leslie, 1996). Mycotoxins produced by *F. proliferatum* include fusaric

acid, gibberellin, moniliformin, the fumonisins, beauvericin, and fusaproliferin (Marasas *et*

al., 1984; Nelson and Juba, 1994; Plattner and Nelson 1994; Ritieni, *et al.*, 1995). Some

strains of *F. moniliforme* consistently produced FB_1 as their major FB constituent but other

strains of *F. proliferatum* did not (Nelson *et al.*, 1994). *Fusarium proliferatum* strains from

the Fusarium Research Center Collection (University Park, PA) found to produce FBs (in

order of highest to lowest) were M-5991 and M-6293 for FB_1 production; M-6104, M-6293.

and M-5991 for FB_2 production; and M-6284, M-6290, and M-6293 for FB_3 production. The

mycotoxin produced by *Alternaria alternata* f.sp. *lycopersici*, AAL-toxin, is structurally

similar to FBs and may be produced by the same biosynthetic mechanisms that *F. moniliforme* used to produce FBs (Gilchrist *et al.*, 1992).

An alternate method for classification based on mating populations within *G. fujikuroi* has been reported by Leslie *et al.* (1992). *Gibberella fujikuroi* has 6 different mating populations, A through F, all within *Fusarium* section Liseola. Of 25 unique strains of *F. moniliforme* isolated from corn kernels associated with ELEM, all were mating population 'A' and produced FB_1 (Leslie *et al.*, 1992). However, FB_1 production by *F. moniliforme* was not required for pathogenicity on maize seedlings (Desjardins *et al.*, 1995). The 'D' mating population of section Liseola is comprised of *F. proliferatum* and produces FBs. Other *Fusarium* species have been reported to produced FBs such as *F. subglutinans* which is in the 'B' mating population (Leslie *et al.*, 1996).

The *Fusarium* fungi are natural soil contaminants and exist worldwide. They are responsible for causing many corn diseases. The FB_1-producing fungus reported most frequently from shelled corn from the United States of America (USA) is *F. moniliforme* (Kommedahl and Windels, 1981). This fungus causes ear rot, seedling blight, root rot, stalk rot, and storage decay in maize. Tseng *et al.* (1995) cultured *F. moniliforme* strains from Taiwan on corn, sorghum, rice, and sugarcane, resulting in the production of FB_1 and FB_2.

In summary, *F. moniliforme* and *F. proliferatum* are wide-spread in soil and produce FBs, along with other mycotoxins, on a major food staple: corn.

2. Occurrence of *Fusarium* fungi and FBs in foods

Strains of *F. moniliforme* have been isolated from various human food commodities including corn, asparagus, watermelon, and vanilla (Bars *et al.*, 1994). Gilchrist *et al.* (1992)

isolated *F. moniliforme* from ripe tomato fruits with symptoms of tomato black mold and

from ears of corn with symptoms of pink ear rot. Fumonisin-producing strains of *F.*

moniliforme are present in many food commodities, such as corn, destined for human

consumption. Bullerman and Tsai (1994) isolated *Fusarium* fungi from the USA during

1991 and 1992 in yellow and white dent corn, popcorn, and sweet corn. The percentages of

infected kernels from the dent corn and popcorn ranged from 8.4-36.2% and 1.8-10.3%,

respectively. Fresh cob sweet corn were infected by *Fusarium* fungi to a lesser extent with a

mean percentage of kernel infection of 0.3%, whereas frozen sweet corn kernels had a mean

percentage of infected kernels of 8.0%. Bullerman and Tsai (1994) reported that the germ

and bran of milled dent corn had the highest *Fusarium* infection and the flaking grit was the

lowest, suggesting that the fungi may concentrate in the germ area and just under the

pericarp. They suggested that the low levels of *Fusarium* contamination in flaking grit

confirmed the findings by Stack and Eppley (1992) that flake cereal has not been associated

with high levels of FBs. Good quality corn kernels meant for human consumption contained

low levels of FBs while other corn material such as damaged corn, cob parts, stalk parts and

screenings contained 74-770 μmoles of FBs/kg solids (Bullerman and Tsai, 1994). Strains of

F. moniliforme were isolated from corn or sorghum from Indonesia, Philippines, and

Thailand. The *F. moniliforme* cultures produced up to 203 μmoles of FB_1 and 62 μmoles of

FB_2/L of LM (Miller *et al.*, 1993). Visconti and Doko (1994) cultured *F. moniliforme* strains

isolated from corn, sorghum, wheat, barley, and mixed feed in Italy, Spain, Poland, and

France on SCM and produced up to 5.7 mmoles of FB_1/kg SCM. The isolates from corn

produced the highest amounts of FB_1 followed by isolates from wheat, barley, and sorghum.

These data suggested that Europe and the USA had a similar pattern of FB-producing *Fusarium* stains from different substrates.

Murphy *et al.* (1993) reported that corn screenings had 10-fold greater amounts of FBs than intact corn isolated from Iowa, Wisconsin, and Illinois during 1988 to 1990. The highest levels of FB_1 were found in corn from the 1989 crop year which contained 53 μmoles FB_1/kg solids. Levels of FB_2 were 50-fold less than FB_1, while levels of FB_3 were 190-fold less than FB_1 in the corn samples from the 1989 crop year but both were linearly correlated with FB_1 concentration. Thiel *et al.* (1992) found 0-3.9 μmoles FB_1 and 0-1.5 μmoles FB_2/kg solids of commercial corn products meant for human consumption in the USA. Trucksess *et al.* (1995) analyzed USA commercial canned and frozen sweet corn samples for FB_1. Out of 97 samples, 35 contained up to 0.1 μmoles of FB_1/kg corn wet weight (wt). One canned corn and one frozen corn sample contained 0.3 and 0.5 μmoles of FB_1/kg corn wet wt, respectively. Hopmans *et al.* (1993) reported 23 to 117 μmoles FB_1/kg of human corn containing foods. In comparison of white and yellow cornmeal, canned yellow corn, tortilla chips, and masa, the highest levels of FB_1 were found in yellow corn meal.

Rheeder *et al.* (1994) found South African corn being shipped to Taiwan in 1989 to contain up to 1.2 μmoles FB_1/kg solids. Other mycotoxins found included FB_2 and moniliformin at less than half the concentration of FB_1. Ueno *et al.* (1993) found FB_1 and FB_2 present in corn kernels, gluten feeds, corn grits, and corn-based products in Asian countries during 1988-1992. The highest levels of these mycotoxins were found in corn kernels imported from the USA in 1988 which contained up to 5.7 and 14.4 μmoles of FB_1 and FB_2/kg solids, respectively. Two out of 7 of the FB_1 containing USA kernel samples also

contained aflatoxin B_1. The coexistence of FBs and aflatoxin is of particular concern because

FB_1 is a promotor and aflatoxin is an initiator of cancer (Gelderblom et al.. 1988; Ellis et al..

1991). Chu and Li (1994) found 49 mmoles of FB_1/kg solids from corn samples collected

from households of counties of Cixian and Linxian of the People's Republic of China. There

was no visible mold contamination on the corn samples. Other commodities have been

shown to contain low levels of FBs including beer, Italian corn grits, USA blue corn meal.

and self rising white meal (Scott and Lawrence, 1995; Shephard et al., 1996).

Companion animal foods have also been reported to contain FBs. Hopmans et al.

(1993) reported 303 to 1956 μmoles FB_1/kg pet food. The highest levels of FB_1 were found

in dog food. Shephard et al. (1996) reported 458 μmoles of FB_1/kg swine feed and up to 100

μmoles of FB_1/kg horse feed.

The FBs are in most corn products to some extent. This is important because the

potential exists that relatively high levels of FBs could exist in human or animal foods.

3. Detoxification of FBs

Detoxification of aflatoxin-contaminated corn can be achieved by treating the corn

material with ammonia (Brekke et al., 1977). However, detoxification of aflatoxin by

ammoniation is not approved for interstate commerce by the FDA. Norred et al (1991)

incubated SCM which contained FB_1 produced by F. moniliforme MRC-826 with ammonium

hydroxide at 50°C for 4 d which reduced the FB_1 content in the SCM by 50%, but the toxicity

to rats was not reduced nor was HFB_1 measured (Norred et al., 1991). Neither increasing the

concentration of ammonia to 5% nor decreasing the duration of the ammonia treatment from

4 to 1 d affected the final concentration of FB_1. The detectable FB_1 levels increased 5-fold

within 4 d following the ammoniation treatment, which suggested that the FB_1 may have been reversibly bound to the SCM matrix. Park *et al.* (1992) treated FB_1-containing SCM with ammonia resulting in 79% reduction in the FB_1 content without production of mutagenic compounds. The SCM was found to contain 120 µmoles FB_1/kg SCM before being exposed to 2% ammonia for 60 min at 15% moisture, 60 pounds per square inch (psi), and 20°C and at 13% moisture, 17 psi, and 125°C. However, the proposed detoxification of FB_1 was not confirmed by feeding laboratory animals nor was the SCM analyzed for HFB_1. Voss *et al.* (1992) found that FB_1 was reduced but not detoxified in ammoniated SCM. The SCM was cultured with *F. moniliforme* MRC 826 and contained 140 µmoles FB_1/kg SCM. After treating with 2% ammonia at 50°C for ≥1 d and dried, the SCM was found to contain 29% less FB_1. The ammoniated and non-ammoniated SCM increased serum ALT, AST, AP, and GGT activities when fed to rats. Ammonia treatment of FB_1 in SCM was not an effective method for its detoxification.

Nixtamilization is the process for masa and tortilla flour production involving alkaline cooking and steeping. This process converts FB_1 to HFB_1 (Hendrich *et al.*, 1993). Hendrich *et al.* (1993) mixed FB-containing corn kernels with clean corn to contain 69 µmoles of FB_1/kg of corn. The FB_1 was produced by *F. proliferatum* M-5991 in SCM. The corn kernels were nixtamilized by heating with 1.2% $Ca(OH)_2$ at 80-100°C for 60 min, steeped overnight, and washed. The nixtamilized corn was as hepatocarcinogenic as the non-nixtamilized corn in diethylnitrosamine (DEN) initiated male Fisher rats fed nutritionally adequate diets. However, nutritionally inadequate diets containing nixtamilized corn produced fewer adenomas than non-nixtamilized corn in rats.

Dupuy et al. (1993) and Bars et al. (1994) found SCM containing FB_1 to be relatively resistant to drying of corn. The $t_{1/2}$ of 2.1 mmoles FB_1 in SCM were 10, 38, 175, and 480 min when heated at 150, 125, 100, and 75°C, respectively. The FB_1 was produced by F. moniliforme in SCM. However, these SCMs were not analyzed for amine blocked-FB_1 or for toxicity. Due to the high required temperature and long heat exposure time, thermal treatment may not be practical for detoxification of FB_1 in SCM. Bordson et al. (1995) added 10 ml of H_2O to 10 g of corn samples containing 22 μmoles of FB_1 and 6.2 μmoles of FB_2 at 110°C for 24-h resulting in a 93% reduction in detectable FB_1 and a 66% reduction in FB_2. Similarly dried pelleted mixed feed samples resulted in undetectable FB_1 and a 44% reduction in FB_2. However, these samples were not analyzed for toxicity or for the presence of amine blocked-FB_1. By heating FB_1 and FB_2 in a pH 4 buffered solution at 150°C for 60 min, Jackson et al. (1996a and 1996b) reduced the levels of FB_1 by 90% and the levels of FB_2 by 70%. However, HFB_1, HFB_2, partially HFB_1, ($PHFB_1$), and $PHFB_2$ were formed. The decomposition rate of FB_1 was pH dependent with a greater rate at pH 4 than pH 7 or 10. At pH 7 and 10, FB_1 was reduced by 20 and 50%, respectively.

The nonenzymatic browning reaction may block the amine group of FB by forming a Schiff's base with a reducing sugar. Murphy et al. (1995) incubated 69.3 μmoles of FB_1 with 100 mM fructose or glucose and 50 mM potassium phosphate, pH 7.0, at 80°C for 48-h which resulted in a 90% decrease in the detectability of FB_1. Lu et al. (1997) reported FB_1-FRU did not induce liver cancer in DEN initiated rats suggesting that FB_1 was detoxified.

The FBs have not been shown to be metabolized by enzymes. Murphy et al. (1996) incubated FB_1 with monoamine oxidase, diamine oxidase, L-amino acid oxidase, and D-

amino acid oxidase without changing the detectability of FB_1 by OPA-derivatization.

Cawood et al. (1994) incubated FB_1 with microsomal enzyme preparations without the formation of FB metabolites suggesting that FB_1 was not metabolized by the esterases or by the cytochrome P-450 monooxygenase enzymes.

The FBs are relatively stable to the temperatures and treatments associated with foods. However, TCA groups can be hydrolyzed or the amine group blocked on the FB backbone potentially resulting in the formation of other toxic or non-toxic compounds.

C. Growth Parameters of *Fusarium* Fungi

1. Solid growth media

Fusarium moniliforme and *F. proliferatum* produce FBs when cultured on solid corn or rice media. Gilchrist et al. (1992) inoculated 1.5 ml H_2O/g SCM or solid rice medium (SRM) with *F. moniliforme* which produced 4.2 mmoles FBs/kg dry wt (DW) of SCM and 6.7 mmoles of FBs/kg DW of SRM. Desjardins et al. (1994) inoculated 50 g of corn and 11 ml H_2O with *F. moniliforme* which produced 8.0 mmoles FB_1 and 4.4 mmoles FB_2/kg DW of SCM. Nelson et al. (1994) inoculated 500 g corn and 500 ml dH_2O with *F. proliferatum* M-5991 which produced 8.0 mmoles FB_1/kg DW of SCM. Optimal moisture content of the SCM for FB_1 production by *F. moniliforme* was found to be 32% (Bars et al., 1994). These data suggested that *F. moniliforme* or *F. proliferatum* may produce up to 8.0 mmoles FB_1/kg DW of SCM or 6.7 mmoles FB_1/kg DW of SRM.

Fungal mutations has been a problem with *F. moniliforme* when grown on a medium rich in carbohydrates (Nelson, 1992). Mycelial or pionnotal type mutants often lost virulence

and the ability to produce toxins. To avoid mutations. cultures were initiated from single

conidia, grown in a low carbohydrate medium, and preserved by lyophilization or in liquid

nitrogen.

2. Liquid growth medium

Fusarium moniliforme and *F. proliferatum* also produce FBs when cultured in LM.

However, concentrations of FBs in LM are usually expressed in wet wt rather than DW

which may result in an apparent lower mass. Jackson and Bennett (1990) inoculated 500 ml

of a basal-salts vitamin-supplemented LM with *F. moniliforme* Northern Regional Research

Center Peoria, IL (NRRL)-13616 which produced 0.1 mmoles FB_1/L of LM after 29 d. The

basal-salts consisted of 3.5 g NH_4SO_4, 2.0 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 16 mg

$MnSO_4 \cdot H_2O$, and 90 g glucose/L of dH_2O. The vitamin-supplement consisted of 500 μg each

of thiamin, riboflavin, pantothenate, niacin, pyridoxamine, and thiotic acid/L of dH_2O. The

culture was grown in a 1 L Erlenmeyer flask on a rotary shaker at 220 revolutions per min

(rpm) and 28°C and the pH was maintained at 5.0 using 2N HCl or 2N NaOH. Lebepe-

Mazur (1993) inoculated 100 ml of basal-salts vitamin-methionine-supplemented LM with *F.*

proliferatum M-5991 which produced 0.5 mmoles FB_1/L of LM after 24 d of incubation.

The vitamin-methionine-supplement consisted of 50 μg each of folate, biotin, and vitamin

B_{12} and 250 mg of dl-methionine/L of dH_2O. The cultures were incubated in 1 L screw

capped flat bottles laid on their sides while shaking at 5 rpm on a rotary shaker without pH

adjustment. These data suggested that adjusting the pH of the LM may result in reduced

production of FBs by *F. moniliforme* or *F. proliferatum*.

Blackwell *et al.* (1994) inoculated 50 ml of LM with *F. moniliforme* NRRL-13616

which produced 0.5 mmoles FB_1/L of modified Myro LM. The modified Myro LM consisted

of 1.0 g $(NH_4)_2HPO_4$, 3.0 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 5.0 g NaCl, 40 g sucrose, and 10 g

glycerin/L of H_2O. Production of FB_1 in 250 ml Erlenmeyer flasks was greatest when the

inoculum was increased from 4.0 to 7.0% (v/v) or the volume of LM was increased from 50

to 75 ml. Miller *et al.* (1994) inoculated 9.3 L of the same LM in a 15 L stirred jar vessel

with *F. moniliforme* NRRL-13616 which produced 0.4 mmoles FB_1/L of LM after 10 d. Air

was pumped into the fermentation vessel at 3.2 L/min. In the fermentation vessel, CO_2

production decreased and reducing sugars were consumed as the FB levels increased

suggesting that FB production occurred during low respiration by *F. moniliforme*. The cell

density of the *F. moniliforme* culture increased from 0 to 9.0 g DW solids/L after 10 d of

incubation. The concentration of FB_1 produced, based on DW solids, was 40 mmoles FB_1/kg

DW solids. These data indicated that there was a 5-fold increase in FB_1 produced by *F.*

moniliforme in LM than in SCM or SRM.

Production of FBs from LM is better than from SCM or SRM as indicated by the FB

concentrations based on DW. The SCM and SRM contained more solids than the LM which

may have hindered the purification procedure of FBs.

D. Fumonisin Analysis

1. Fluorometry

The structure of FBs does not allow for its direct detection using most analytical

detectors such as an ultraviolet absorbance detector. Like sphingosine and amino acids, the

primary amine group of FBs can be derivatized with fluorescent compounds and quantified

using a fluorometric detector. Analysis of FBs initially involves a clean-up procedure

followed by derivatization with a fluorescent molecule such as o-pthaldialdehyde (OPA) and

a reducing agent such as 2-mercaptoethanol (MCE) followed by C_{18}-HPLC with fluorometric

detection. Previous work by Merrill *et al.* (1988) analyzed sphingosine, which is structurally

similar to FBs, by derivatization with OPA containing MCE for at least 5 min, loading onto a

C_{18}-HPLC column, elution with MeOH:5.0 mM KH_2PO_4 at pH 7.0 (90:10), and

quantification using a fluorometer with an excitation wavelength (λex) of 340 nm and an

emission wavelength (λem) of 455 nm. Similarly, FB_1 and FB_2 were derivatized with OPA

containing MCE for 1 to 2 min, loaded onto a C_{18}-HPLC column, eluted with MeOH:0.1 M

NaH_2PO_4 (80:20) pH 3.3, and quantified using a fluorometer with an λex of 335 nm and an

λem of 440 nm (Shephard *et al.*, 1990). This procedure has been accepted, with slight

modification of mobile phase to MeOH:0.1 M NaH_2PO_4 (77:23) pH 3.3, as an A.O.A.C.

Official Method 995.15 for quantification of FB_1, FB_2, and FB_3 that have been extracted and

cleaned-up from corn (Sydenham *et al.*, 1996b). Rice and Ross (1994) reported FB_1, FB_2,

and FB_3 were derivatized with OPA and MCE for 10 min, loaded onto a C_{18}-HPLC column,

eluted with a ACN:50 mM KH_2PO_4 at pH 3.3 (40:60), and quantified using a fluorometer

with an λex of 335 nm and an λem of 440 nm. Poling and Plattner (1996) reported FB_3 and

FB_4 were derivatized with OPA and MCE for 1 to 2 min, loaded onto a C_{18}-HPLC column,

and eluted with MeOH:H_2O:0.1 M NaH_2PO_4 adjusted to pH 3.35 A (60:40) and B (80:20) at

25% A for 1 min, a linear gradient to 100% B for 15 min, and 100% B for 5 min. The OPA-

FB derivatives were quantified using a fluorometer with an λex of 334 nm and an λem of 440

nm. Thakur and Smith (1996) modified the conventional HPLC procedure for separation of the OPA-FB derivatives by leaving the buffering-salts out of the mobile phase. Samples containing FB_1, FB_2, HFB_1, HFB_2, and partially hydrolyzed FB_1 ($PHFB_1$) were derivatized with a commercial reagent OPA-reducing agent kit for 1 min, loaded onto a double-end-capped C_{18}-HPLC column, eluted with $ACN:H_2O$:acetic acid (40:59:1) and (60:39:1) gradient 0% to 100% of the latter phase in 9 min. The OPA-FB derivatives were quantified using a fluorometer with an λex of 255 nm and an λem of 442 nm. This HPLC mobile phase for FB separation was reported to be better than the conventional buffering-salts mobile phase because it was more appropriate for on-line liquid chromatography (LC)-MS analysis and required less time for column equilibration. Miyahara *et al.* (1996) reported FB_1 and FB_2 were derivatized with OPA containing N-acetyl-L-cysteine in an in-line reaction coil and loaded onto an ion pair LC. This derivatization procedure produced a more stable derivative than the OPA containing MCE procedure (Miyahara *et al.*, 1996). The OPA-FB derivatives were quantified using a fluorometer with an λex of 336 nm and an λem of 460 nm.

Holcomb *et al.* (1993) reported that FB_1 was derivatized with (9-fluorenylmethyl) chloroformate (FMOC) for 30 sec followed by pentane extraction, loaded onto a C_{18}-HPLC column, and eluted with MeOH:citrate buffer A (30:70) and B (70:30) at a linear gradient from 10 to 40% B over 9 min followed by a linear gradient to 100% B in 1 min. The FMOC-FB derivatives were quantified using a fluorometer with an λex of 263 nm and an λem of 313 nm.

Ware *et al.* (1993) compared amine derivatizing reagents, naphthalene-2,3-dicarboxaldehyde (NDA), OPA, phenyl isothiocyanate, fluorescamine, and

nitrobenzoxadiazole, for their stability after derivatization with the amine group on FB_1. The derivatizing reagent with the best stability was NDA, however, it involved a complex procedure of heating FB_1, NDA, and cyanide at 60°C for 15 min. However, the NDA-FB_1 derivative retained 90% of its fluorescence after 10-h while the OPA-FB_1 derivative retained only 33%. Phenyl isothiocyanate and fluorescamine were less effective in their ability to form a stable derivatized FB_1 compound. The NDA-FB_1 derivative was loaded onto a C_{18}-HPLC column, eluted with ACN:H_2O:acetic acid (55:45:1), and quantified using a fluorometer with an λex of 410 nm and an λem of 440 nm.

Rottinghaus *et al.* (1992) developed a rapid procedure screening samples for the presence of FB compounds. C_{18}-Thin layer chromatography (TLC) plates were spotted with FB_1 and FB_2, developed with $MeOH$:4% aqueous KCl (3:2), and sprayed with fluorescamine. The fluorescamine derivatized with the amine group of FB_1 and FB_2. After drying the fluorescamine-FB compounds were observed under long-wave ultraviolet (UV) light as bright yellowish-green bands.

The OPA-FB_1 derivatization method has been the method of choice for FB identification and quantification by researchers. This method is relatively simple, cheap, and reproducible. The NDA-FB_1 derivative was shown to be more stable than OPA-FB_1 but appeared to be more a complicated procedure. Identification of FBs by TLC is relatively simple, cheap, and quick but, is not useful for quantification of FBs.

2. ELISA

Detection of FBs by an enzyme-linked immunosorbant assay (ELISA) involves monoclonal or polyclonal antibodies raised against FBs. When corn and corn based foods

were quantified for FB_1 by monoclonal antibody competitive direct-ELISA. greater levels of

FB_1 were obtained compared to OPA-FB_1 fluorometric detection (Pestka *et al.*, 1994; Tejada-

Simon *et al.*, 1995; Sydenham *et al.*, 1996a; Sutikno *et al.*, 1996). Similar results were

observed by Shelby *et al.* (1994) in FB_1 quantified in corn using monoclonal antibody

competitive indirect-ELISA compared to TLC. These data suggested that compounds that

were structurally related to FBs were detectable only by an ELISA.

Yeung *et al.* (1996) quantified the amount of FB_1 in corn by polyclonal antibodies

CD-ELISA (PA-CD-ELISA) but did not compare their results to other FB_1 detection

procedures. Schneider *et al.* (1995) developed a qualitative 10 min detection method for FB_1

using a noncompetitive anti-FB_1 antibodies enzyme linked immunofiltration assay.

Compared to fluorometric analysis, the ELISAs have the potential to provide quick

results where a large number of samples can be qualitatively screened for FBs at one time.

These ELISAs are applicable for field and industry use. However, due to cross reactivity

with other FBs and structurally related compounds, the amounts of FBs detected in corn and

food samples by ELISAs have been greater than the amounts detected by conventional

HPLC-OPA derivatized methods.

3. Other analytical methods

Due to the structure of FBs, their direct detection by UV detectors has not been

reported. Alberts *et al.* (1993a) derivatized FB_1, FB_2, and FB_3 with maleic anhydride and

quantified them using HPLC with an UV detector at 240 nm. However, the FB-maleyl

derivatives had low end detection limit of 112-140 μmoles FBs/g of SCM compared to 1-18

nmoles FBs/g SCM for OPA-ME analysis (Sydenham *et al.*, 1996) and 55-83 pmoles FBs/g

SCM for ELISAs (Schneider *et al.*, 1995). Wilkes *et al.* (1995) quantified FBs with

evaporative light-scattering detection (ELSD) after HPLC separation without derivatization.

However, the ELSD could only detect about 1.7 μmoles FBs/g of SCM and the standard

curves are not linear. Due to its non-invasive analysis of FBs, the ELSD was practical for

semi-preparative purification of FBs. The method of FB detection by silica-TLC

(Ackermann, 1991) was enhanced by a spectrophotodensitometer detector (Bars *et al.*, 1994)

making silica-TLC analysis of FBs applicable for quantitation having a low end detection

limit of 42-70 mmoles FBs/g of SCM and 4-7 mmoles FBs/g of purified extracts.

Other methods that have reported to characterize FBs by mass spectrometry (MS)

include continuous flow fast atom bombardment and ion spray-MS (Mirocha *et al.*, 1992),

electrospray-MS (Poling and Plattner, 1996), negative-ion thermospray-MS (Thakur and

Smith, 1994), and particle-beam-MS (Young and Lafontaine, 1993).

E. Fumonisin Clean-Up

1. Solid matrices

For quantification in corn and corn-based foods, FBs need to be cleaned-up so they

can be identified using a detection method. Hopmans and Murphy (1993) cleaned-up FB_1,

FB_2, FB_3, and HFB_1 from corn based foods by extraction with 50% ACN and elution from a

C_{18}-SPE cartridge. Rice *et al.* (1995) cleaned-up FB_1, FB_2, and FB_3 from corn, poultry feed,

and SCM by extraction with 50% ACN and elution from a C_{18}-SPE cartridge. Miyahara *et*

al. (1996) cleaned-up FB_1 and FB_2 from corn and corn-based foods by extraction with 50%

ACN and elution from C_{18}-SPE and strong anion exchange (SAX)-SPE cartridges.

Sydenham *et al.* (1996b) cleaned up FB_1, FB_2, and FB_3 from corn by extraction with 75%

MeOH and elution from a SAX-SPE cartridge. This last procedure has been accepted as an

AOAC Official Method as a pre-quantification clean-up procedure for determination of FB_1.

FB_2, and FB_3 levels in corn (Sydenham *et al.*, 1996b). However. HFBs will not be retained

on the SAX-SPE column and are missed by the AOAC method. Ware *et al.* (1994) cleaned-

up FB_1 and FB_2 from corn by extraction with 80% MeOH and elution from an

immunoaffinity Fumonitest-LC column. Selim *et al.* (1996) cleaned-up FB_1 from corn. corn

dust. and SCM by extraction with supercritical CO_2 (SC-CO_2). The SC-CO_2 fluid extraction

removed 40-fold more FB_1 from the corn and corn dust, was faster, and was more

reproducible than extraction with ethyl acetate 2 times followed by 75% MeOH 3 times.

Clean-up procedures are similar to purification procedures only on a smaller scale with the

use of cartridges rather than columns.

F. Fumonisin Purification

1. Solid matrices

Purification of FBs is the first step in FB quantification and usually involves

extraction from a solid matrix with a solvent, such as MeOH or ACN, followed by

chromatography and finishing with purified dry solids for weight determination. Cawood *et*

al. (1991) purified FB_1, FB_2. FB_3, and FB_4 produced by *F. moniliforme* MRC-826 on SCM

by extraction with 75% MeOH and elution from XAD-2-, silica gel-. and C_{18}-LC to obtain

>90% pure FBs with 40% yield. Meredith *et al.* (1996) purified FB_1 produced by *F.*

moniliforme MRC-826 from SRM by extraction with 50% acetonitrile (ACN) and elution

from a C_{18}-LC column and 2 cyano-LC-cartridges to obtain $\geq 95\%$ pure FB_1 with a recovery

of 77%. Poling and Plattner (1996) purified FB_3 and FB_4 to >90% FB_3 and FB_4 with >95%

recovery produced by *F. moniliforme* from SCM by extraction with 50% ACN. elution from

a NH_2-solid phase extraction (SPE) cartridge with 5% acetic acid in MeOH. and elution from

a C_{18}-SPE cartridge with increasing concentrations of ACN.

Monomethyl and dimethyl esters of FB_1 and FB_2, formed as artifacts from extractions

of FBs from SCM with MeOH, may interfere with the purification of FB_2. FB_3, and FB_4

(Gelderblom *et al.,* 1992). Monomethyl and dimethyl esters have not been reported to be

produced during extraction of FBs with acetonitrile (ACN). In addition, the extraction of

FB_1, FB_2, and FB_3 from SCM with 50% ACN was 3-fold greater than 75% MeOH after 8-h

(Nelson *et al.,* 1994; Rice *et al.,* 1995). Due to the reported formation of artifacts and better

ability to extract FBs from SCM, ACN may be a better solvent than MeOH for FB extraction

from SCM.

Complicated matrices such as biological tissues require the use of a chelating agent

such as ethylenediaminetetraacetic acid (EDTA) to extract impurities associated with the

FBs. Shephard *et al.* (1994b) cleaned-up FB_1 from primate feces by extraction of impurities

with 0.1 M EDTA 6 times and elution of the FB_1 from a C_{18}-SPE cartridge. Hopmans *et al.*

(1997) cleaned-up FB_1, HFB_1, and FB_1-FRU from rat feces by extraction of the impurities

with 0.1 M EDTA 9 times and elution from a C_{18}-SPE cartridge.

The FBs have been purified from solid matrices by extraction and LC to $\geq 95\%$ purity

and up to 95% recovery.

2. Liquid matrices

Purification of FBs from liquid matrices does not require solvent extraction like that of solid matrices. In addition, the liquid matrices do not apparently contain as many lipophilic compounds as solid matrices which co-purify with FBs during LC (Jackson and Bennett, 1990). Miller *et al.* (1996) purified FB_1 produced by *F. moniliforme* NRRL-13616 in LM to 97% purity with 89% yield by ion-exchange, silica gel, and C_{18}-LC. Lebepe-Mazur (1993) purified FB_1 produced by *F. proliferatum* M-5991 in LM to >90% purity with 40% yield by XAD-2, silica gel, and C_{18}-LC. The use of ion-exchange to purify FBs may result in increased purities of FB_1.

G. Isotope Labeling of Fumonisin B_1

1. Deuterium labeling

Labeling FB with isotopes, such as deuterium (^2H), can be helpful for understanding the production of FB by *F. moniliforme* and identify possible radiolabeling substrates. Isotope or radiolabeled FB_1 is needed for animal toxicity studies. Plattner and Shackelford (1992) and Plattner and Branham (1994) added $[^2H_3]$-(CH_3)-L-methionine to LM cultured with *F. moniliforme* M-2326 resulting in the production of $[^2H_3]$-$(C$-12, 16)-FB_1 (Fig. 1). Three or 6 ^2H atoms were incorporated into FB_1 suggesting that the CH_3 group of methionine was incorporated into one or both of the CH_3 groups at the C-12 and C-16 positions on the FB_1 backbone. Methionine, a substrate used by many fungal methyl transferases (Plattner and Shackelford, 1992), stimulated the production of FB_1 when added to LM cultured with *F.*

moniliforme and incubated for 13 d. These data suggested that methionine could be used as a substrate for radiolabeling FBs.

2. ^{13}C Labeling

Alberts *et al.* (1993b) added [^{13}C]-(CH$_3$)-L-methionine to SCM cultured with *F. moniliforme* MRC-826 which resulted in the production of [^{13}C]-(C-12, 16)-FB$_1$ supporting the observation of Plattner and Shackelford (1992) and Plattner and Branham (1994), that methionine supplied the CH$_3$ groups located on the C-12 and C-16 positions of the FB$_1$ backbone. Blackwell *et al.* (1994) added [^{13}C]-(C-2)-acetate to LM cultured with *F. moniliforme* NRRL-13616 resulting in the production of [^{13}C]-(C-4, 6, 8, 12, 14, 16, 18, 20)-FB$_1$. When [^{13}C]-(C-1)-acetate was added to LM cultured with *F. moniliforme* NRRL-13616, [^{13}C]-(C-3, 5, 7, 9, 11, 13, 15, 19)-FB$_1$ and a [^{13}C]-carboxyl in the TCA groups were identified. When [^{13}C]-(CH$_3$)-L-methionine, [^{13}C]-(C-5)-L-glutamate, and [^{13}C]-(C-3)-serine were added to LM cultured with *F. moniliforme* NRRL-13616, [^{13}C]-(C-12, 16)-FB$_1$, [^{13}C]-TCA groups, and [^{13}C]-(C-1)-FB$_1$ were identified, respectively. [^{13}C]-(C-3)-L-Alanine was also reported by Blackwell *et al.* (1996) to produce [^{13}C]-(C-1)-FB$_1$. These data suggested that the long chain FB-backbone may have been derived from the condensation of acetyl-CoA units resulting in the even label throughout the FB molecule during polyketide synthesis. Radiolabeled acetate may be a better substrate for radiolabeling FB because more carbons are labeled per molecule of FB.

3. ^{14}C Labeling

Radiolabeling FB with [^{14}C] can be useful for animal toxicity studies. The amount of incorporation of radioactivity that is incorporated into a molecule can be expressed as

specific activity (SA) having the units of milli-Curie (mCi)/mmole Alberts *et al.* (1993b)

added 50 mg L-methionine and 200 µCi [^{14}C]-(CH$_3$)-L-methionine to 30 g of SCM cultured

with *F. moniliforme* MRC-826. The *F. moniliforme* produced [^{14}C]-FB which was purified to

14 mmoles FB/kg DW with a SA of 0.04 mCi/mmole. The ^{14}C-methionine was added during

logarithmic phase of FB$_1$ production from 3 to 11 d of incubation and was incorporated into

the C-21 and C-22 positions of the FB$_1$ backbone. However, when 250 mg sodium acetate

and 125 µCi of [^{14}C]-acetate were added to 30 g SCM containing *F. moniliforme*, the SA of

the purified FBs was only 0.01 mCi/mmole. These data suggested that when 250 mg sodium

acetate were added to 30 g SCM, *F. moniliforme* decreased the production of FB$_1$.

Lebepe-Mazur (1993) incubated 500 ml of LM containing *F. proliferatum* M-5991

for 8 d, added 100 µCi [^{14}C]-(CH$_3$)-L-methionine, and harvested after 12 d. The *F.*

proliferatum produced [^{14}C]-FB$_1$ which was purified to 54 µmoles of 85% pure [^{14}C]-FB$_1$

with a SA of 0.07 mCi/mmole. Norred *et al* (1993) added 1 mCi of [^{14}C]-(CH$_3$)-methionine

to 100 ml of LM cultured with *F. moniliforme* M-2326 and harvested after 10 d of

incubation. The *F. moniliforme* produced [^{14}C]-FB$_1$ which was purified to 55.5 µmoles [^{14}C]-

FB$_1$ (>95% pure) with a SA of 0.74 mCi/mmole. Blackwell *et al.* (1994) added 0.1 mCi [1,2-

^{14}C]-acetate to 50 ml of LM cultured with *F. moniliforme* NRRL-13616/d between 7 and 14 d

of incubation and harvested after 17 d. The *F. moniliforme* culture produced [^{14}C]-FBs which

were purified to 36 µmoles FBs with a SA of 0.65 mCi/mmole. These data suggested

different methods for [^{14}C]-labeling of FBs in LM cultures of *F. moniliforme*.

H. Fumonisin Toxicity

1. History

Prior to 1988, SCM containing cultures of *F. moniliforme* were known to induce ELEM and equine hepatotoxicity, PPE, rat liver cancer, and were associated with human esophageal cancer in South Africa (Marasas, 1996). During 1988 to 1991, the FBs were isolated, characterized, and confirmed to be inducers of ELEM, PPE, and liver cancer in horses, swine, and rats, respectively (Marasas, 1996).

Epidemiological studies on FBs have shown that FBs exist in animal and human food and suggest a correlation between FB consumption and human esophageal cancer. Castella et al. (1996) found that over 97% of *F. moniliforme* strains isolated from poultry feed produced FB_1. Fumonisin B_1 was present in 89% of the corn samples collected from different regions of Costa Rica (Viquez *et al.*, 1996). The FBs are the major mycotoxin contaminants of corn-based foods in Eastern and Southern Africa (Doko *et al.*, 1996). Corn samples, taken from households of Linxian China in areas that had high incidences of human esophageal cancer, were found to contain an average of 103 and 48 μmoles FB_1/kg moldy and non-moldy corn, respectively (Chu and Li, 1994). Although inconclusive, these data suggested a possible correlation between FBs and human esophageal cancer. Chu and Li (1994) suggested that the carcinogenic potential of FBs may be increased by the ability of *F. moniliforme* to produce carcinogenic nitrosamines from nitrate and precursor amines.

Marasas *et al.* (1988) dosed 2 horses (15 years old [y], 425 kg BW; 15 y, 385 kg BW) by gavage with a corn diet containing 3.5 and 1.75 μmoles FB_1/kg BW/d for 6 d, respectively. Both horses developed ELEM without signs of demyelinization. The 3.5

μmoles FB_1/kg BW/d dose induced fatal hepatosis, mild brain lesions, and elevated activities

of aspartate transaminase (AST), gamma-glutamyltransferase (GGT), lactate dehydrogenase

(LD), and total bilirubin (TB) in the horse up to 4 d after the treatment terminated. The 1.75

μmoles FB_1/kg BW/d dose induced mild hepatosis, severe brain lesions, and elevated

activities of AST, GGT, and LD in the horse 3 d after commencement of the treatment. In a

second experiment, ELEM was induced in a horse by intravenous injections (iv) of 0.17

μmoles FB_1 (92 % pure)/kg BW/d for 7 d. The FB_1 used in the iv dose was produced by $F.$

$moniliforme$ MRC-826 on SCM. Biochemical changes in the horse included elevations of

AST and GGT up to 4 d after the treatment terminated. Kellerman et $al.$ (1991) dosed a filly

(0.75 y, 150 kg BW) and a colt (1.2 y, 190 kg BW) by gavage with 12.4 mmoles FB_1 (50%

pure) over a period of 33 d and 1.7 mmoles FB_1 (95% pure) over a period of 29 d,

respectively, which induced ELEM in both horses. Serum AST levels in the filly and serum

GGT levels in the colt were elevated. No other changes in biochemical markers, AST, GGT,

LD, and TB, were observed. The kidneys from each horse were swollen and grayish-yellow

in color. In the final treatments, the horses developed clinical signs typical of ELEM

(including stumbling, apathy, tremors, and inability to eat). The total dose required for the

appearance of the initial clinical signs was 4.1 mmoles FB_1 (50% pure) for the filly and 8.3

mmoles FB_1 (95% pure) for the colt. Indications of ELEM were apparent in the left frontal

lobe of the filly and the left frontal to occipital lobes of the colt. Wilson et $al.$ (1992) fed

corn containing 20.8, 0, 30.5, 0, and 30.5 μmoles FB_1/kg diet/d for 130, 30, 13, 7, and 58 d,

respectively, to a gelding horse (4 y, 152 kg BW) which induced ELEM and death in the

horse. This diet regimen was designed to determine on the minimum dose of FB_1 that was

toxic to this horse. However, the corn also contained 31 µmoles of FB_2/kg corn. Prior to

death, the horse showed typical symptoms of ELEM (aggression, ataxia, and confusion).

During the final treatment, the dosage rate was 0.25 µmoles FB_1/kg BW/d. Within 9 d of

death, the horse experienced increased serum TB, direct bilirubin (DB), alkaline phosphatase

(ALP), GGT, and bile acids. Three other horses of similar age survived the treatments and

were euthanized after 146 d into the final treatment. They did not develop ELEM or

abnormal serum chemistry levels, but showed typical physical symptoms of ELEM and had

histopathological-brain lesions. In a second experiment reported by Wilson *et al.* (1992), 5

horses fed a low fat corn diet containing 2.0 µmoles FBs/d for 122 d followed by a normal

corn diet containing 4 µmoles FBs/d for 58 d. These horses survived the FB_1 treatments

without abnormal serum chemistry levels. However, they showed typical physical symptoms

of ELEM and developed histopathological-brain lesions. These data support the findings that

FB_1, purified or contained in corn, caused ELEM in horses at a minimum level of 0.17 to 5.5

µmoles FB_1/kg BW for 7 d. When dosed by iv, FB_1 was 10-fold more toxic to horses than

when dosed by gavage suggesting that FB_1 was only partially absorbed when consumed by

these horses. These FB-treated horses showed signs of liver or kidney toxicity based on

serum chemistry data.

Harrison *et al.* (1990) fed corn containing 215 µmoles FB_1/kg diet for 7 d to young

swine (16-24 kg BW) which caused PPE and death in the swine. The FB_1 was produced by a

fungus identical in morphology to *F. moniliforme* MRC-826. In a second experiment, a

young pig (7-8 kg BW) dosed by iv with 0.5 µmoles of FB_1 (≥95% pure)/kg BW/d developed

PPE and died after 5 d. Pathological lesions in the lungs were found in the PPE affected pigs

with typical PPE symptoms including the presence of clear foamy liquid in the trachea and

bronchi, air-clotting golden-yellow liquid in the thoracic cavity, and interlobular edema of the

lung. However, in a third experiment reported by Harrison *et al.* (1990), a pig dosed by iv

with 0.24 μmoles of FB_1 (≥95% pure) and a pig dosed by iv with 0.4 μmoles of FB_2 (≥95%

pure)/kg BW/d for 7 d, did not develop PPE. Osweiler *et al.* (1992) fed a corn diet

containing 23 μmoles FB_1/kg diet to weanling pigs (7-15 kg BW) causing PPE after 5 d. The

FB_1 was produced by *F. moniliforme* MRC-3033. Prelusky *et al.* (1996) found FB_1 to

accumulate in the liver and kidneys of pigs (6-8 weeks [wk] old, 9-13 kg BW) fed 4 μmoles

$[^{14}C]$-FB_1/kg diet for 12 d followed by 3 μmoles $[^{14}C]$-FB_1/kg diet for 12 d. The $[^{14}C]$-FB_1

was produced by *F. moniliforme* NRRL-13616. These data suggested that FB_1 may have

caused PPE after these pigs consumed 3 μmoles FB_1/kg diet for 12 d. When pigs were dosed

by iv, FB_1 was almost 20-fold more toxic to pigs than when dosed by gavage suggesting that

FB_1 was not well absorbed by the GI tract of these pigs.

Motelin *et al.* (1994) fed a corn screenings diet which was found to contain 243

μmoles FBs/kg diet to male weanling pigs (6-13 kg BW) inducing PPE after 14 d. However,

no PPE symptoms were observed in pigs fed 139 μmoles FBs/kg diet. These data suggested

that PPE in swine may have been caused by the consumption of diet containing 139-243

μmoles FBs/kg diet. The presence of FB_2, at about 30% of the total FBs, may have played a

role in the toxicity of the FB-containing diet. There has been little work comparing PPE

toxicity of FB_1 and FB_2.

Smith *et al.* (1996) fed pigs a corn diet containing 49 μmoles HFB_1/kg BW/d for 7 d.

The HFB_1 was synthesized from FB_1 produced by *F. moniliforme* MRC-826. After 8 d, the

pigs were catheterized and their right atriums were infused with Monastral Blue or

Pseudomonas aeruginosa to measure the efficiency of pulmonary clearance. This HFB_1 level

may have inhibited pulmonary intravascular macrophages from clearing particulates and

bacteria from the circulation which, could have made these pigs vulnerable to infectious

diseases. These data suggested that HFB_1 is toxic to pigs and could have potentially caused

PPE.

After FB_1 has been identified and purified, it has been reported to cause ELEM and

PPE. However, the mode of toxicity and the ability to target different organs in different

animals, such as the brain of horses and the lungs of pigs, has not been reported. The

implications this has for humans is unclear. So far, FB consumption has been associated with

human esophageal cancer only by epidemiological studies.

2. Alteration of sphinganine and sphingosine metabolism

Fumonisins inhibit ceramide synthase, the main enzyme involved with converting

sphinganine to ceramide (Merrill *et al.*, 1993) during sphingolipid metabolism in mammals.

Once formed, ceramide may then be converted to sphingosine or other sphingolipids. Levels

of sphingosine are only slightly increased in cells exposed to FBs. This may be due to the

breakdown of complex sphingolipids. Therefore, inhibition of ceramide synthase could

result in the accumulation of sphinganine and an increase in the sphinganine/sphingosine

ratio of mammalian cells (Merrill *et al.*, 1993). Riley *et al.* (1993) observed increased

sphinganine levels, an increase in the sphinganine/sphingosine ratio, and a slight increase in

sphingosine in male and female Sprague-Dawley rats (3-5 wk old) fed 20 µmoles FB_1 (>99%

pure)/kg diet for 4 wk. The FB_1 was produced by *F. moniliforme* MRC-826 on SCM. The

kidneys of the male and female rats and urine from the male rats, fed ≥ 20 μmoles FB_1/kg

diet, had significantly higher sphinganine levels than their respective controls. The

sphinganine levels from the urine of female rats were significantly increased when fed ≥ 67

μmoles FB_1/kg diet. Ultrastructural damage was observable in the kidneys from the male rats

fed ≥ 20 μmoles FB_1/kg diet and in female rats fed ≥ 67 μmoles FB_1/kg diet. A similar pattern

of tissue damage was observed in the male and female rat livers. Elevated sphinganine levels

and an increase in the sphinganine/sphingosine ratio observed in the urine of these rats may

have been due to FB_1-induced kidney or liver toxicity. The male rats appeared to be slightly

more sensitive to FB_1 than female rats as perceived by the urine and ultra-structural damage

data. These data supported the theory that FB_1 inhibited ceramide synthase because

sphinganine was increased in rats dosed with FB_1.

Martinova and Merrill (1995) dosed BALB/c mice by iv with 0.028 μmoles of FB_1

(>95% pure)/kg BW/d for 5 d resulting in a 294-fold increase in liver sphinganine and a 6-

fold increase in liver sphingosine. These data suggested that FB_1 produced similar signs of

liver toxicity in mice as those seen in rats.

Weibking et al. (1993) fed 100 μmoles FB_1/kg diet to broiler chicks for 21 d and

observed a 3-fold increase in serum sphinganine levels and a 6-fold increase in the

sphinganine/sphingosine ratio. The FB_1 was produced by F. moniliforme M-1325 on corn

and extracted with acetone:chloroform (75:25). These data suggested that chickens may be

less toxic to FB_1 than rats.

The FB_1 (>95% pure) concentration causing 50% inhibition of ceramide synthase in

mouse brain microsomes (IC_{50}) was 0.075 μM FB_1 (Merrill et al., 1993). Fumonisin B_1 was

shown to competitively inhibit ceramide synthase with sphinganine and stearoyl-CoA as substrates. Mouse brain microsomes produced ^{14}C-sphingolipids when incubated with [^{14}C]-serine. However, when similar mouse brain microsomes were incubated with [^{14}C]-serine and 25 μM FB_1, the amount of [^{14}C]-sphingolipids were decreased. This same dose of FB_1 caused free sphinganine to increase 20-fold within 24-h while no change occurred in sphingosine concentrations from the mouse brain microsomes. Harel and Futerman (1993) treated rat hippocampal neurons with 10 μM FB_1 (>95% pure) *in vitro* resulting in an inhibition of sphingolipid synthesis after 2 to 3 d of incubation. Control neuron cells increased their axonal length from 170 to 240 μm, while no increase in axonal length were observed in FB_1 treated neurons. Furthermore, when ceramide was added to FB_1 treated neurons, an increase in axonal length was observed indicating that FB_1 toxicity to these neurons may be due to inhibition of ceramide synthesis. These data suggested that FB_1 affected sphingolipid metabolism of mouse brain microsomes and the axonal growth of rat neuron cells.

Fumonisin B_1 affects kidneys, livers, and brains of rats and or mice as noted by tissue damage and increases in sphinganine and the sphinganine/sphingosine ratio associated with these organs. These data suggested that FB_1 altered sphingolipid metabolism which may be the mechanism for its toxicity.

3. Liver and kidney toxicity

Animal dosing of FBs, intraperitoneally or orally, may result in different bioavailabilities due to the low absorptions of FBs by the GI tract of mammals. Bondy *et al.* (1996) dosed male Sprague-Dawley rats (120-170 g BW) intraperitoneally with 14 μmoles

FB$_1$ (98% pure)/kg BW/d causing liver and kidney toxicity and a reduction in BW, food

consumption. and feces production after 4 d. Hepatotoxicity was correlated with

histopathology. Nephrotoxicity was correlated with an elevated blood urea nitrogen and with

changes in kidney morphology. In addition. FB$_1$ was toxic to the immune system based on

reduced thymus weight. disseminated thymic necrosis. and elevated serum immunoglobulin

M levels. Martinez-Larranaga *et al.* (1996) administered male Wistar rats (200-210 g BW)

with 0.35 μmoles of FB$_1$ (>95% pure)/kg BW/d for 6 d intraperitoneally which caused an

increase in the activity of microsomal and peroxisomal enzymes. However, no metabolites

of FB$_1$ were found confirming the findings of Cawood (1994) that rat liver enzymes did not

modify FB$_1$. Suzuki *et al.* (1995) dosed male Sprague-Dawley rats (120-170 g BW) with

10.4 μmoles FB$_1$ (98% pure)/kg BW/d for 4 d intraperitoneally resulting in kidney toxicity as

determined by increased urine volume. decreased urine osmolality. proteinuria. enzymuria.

and ion transport. These data suggested that FB$_1$. administered orally or intraperitoneally.

may induce liver and kidney toxicity in rats. Rats dosed intraperitoneally with FB$_1$

developed an acute toxicity to FB$_1$. However. rats dosed by gavage were not acutely

intoxicated. suggesting that very little of the FB$_1$ may have been absorbed by the GI tract of

these rats.

Gelderblom *et al.* (1991) fed 69 μmoles FB$_1$ (≥90% pure)/kg diet to male BD-IX rats

(70-80 grams [g] BW) for 26 months causing kidney toxicity in the rats. The FB$_1$ was

produced by *F. moniliforme* MRC-826. Elevated serum levels of AST. glutamine serum

transferase (GST), and TB were observed in the rats. However. serum levels of ALT and

ALP were not elevated. Other toxic effects (esophageal. heart, and fore-stomach lesions).

previously associated with rats fed corn diets containing FBs were not observed. Osweiler *et al.* (1992) reported subacute hepatotoxicosis with hepatocellular necrosis, hepatomegalocytosis, and an increased number of mitotic figures in livers of weanling swine (7-15 kg BW) fed a corn diet containing 167 μmoles of FBs/kg diet for 6 d. Similar results of hepatotoxicosis occurred in 2 other weanling swine (7-15 kg BW) dosed by iv with 1.1 μmoles of FB_1 (98 % pure)/kg BW/d for 14 d. These swine became cachextic and, after 12 d, icterus (jaundice) was obvious in the skin, sclera (white portion of the eyes), and oral mucous membranes. Haschek *et al.* (1992) administered 11 μmoles of FB_1 (>95% pure)/kg BW/d for 9 d to a pig (4 wk old) by iv causing liver (hepatocyte disorganization and necrosis) toxicity. Similar findings resulted in another pig (4 wk old) dosed by iv with 6.4 μmoles FB_1 (>95% pure)/kg BW/d for 4 d. Fumonisin B_1 caused an increase in the activity of serum liver enzymes (AST, ALP, GGT), TB, and cholesterol resulting in liver toxicity in these animals.

Motelin *et al.* (1994) fed a corn diet, that was found to contain 139 μmoles of FBs/kg diet, for 14 d to male weanling pigs (6-13 kg BW) which induced liver toxicity. Increases in serum bilirubin and cholesterol concentrations and in GGT, ALP, alanine amino transferase (ALT), AST, and arginase (ARG) activities were observed in the pigs. Based on regression analysis of the 14 d serum chemistry profiles (with ALP being the most sensitive), the NOEL for liver toxicity in the FB fed swine was ≤17 μmoles of FBs/kg diet. Liver enzyme biomarkers may be a more sensitive assay for FB toxicity than adverse toxic effects in swine after consumption of FBs. For instance, the NOEL which Motelin *et al.* (1994) found to cause PPE in swine fed corn screenings for 14 d was 243 μmoles of FBs/kg diet. However,

the FBs consumed by the swine may have induced biomarkers of liver toxicity when the swine consumed ≤17 μmoles of FBs/kg BW.

Edrington *et al.* (1995) intraruminally gavaged corn containing 15 μmoles of FBs/kg diet/d to crossbred Suffolk × Rambouillet wether ruminant-lambs (32 kg BW) for 4 d which induced liver damage. The blood serum liver enzyme (ALP, GGT, AST, and LDH) activities and liver and kidney damage indicators (cholesterol, triglycerides, urea nitrogen , and creatinine) were increased in the FB treated lambs compared to control lambs. However, lambs developed more severe toxic symptoms such as tubular nephrosis, mild hepatopathy, diarrhea, lethargy, decrease in feed intake, and death, when gavaged with corn containing 61 μmoles of FBs/kg diet/d for 4 d. Osweiler *et al.* (1993) reported increased activity of serum enzymes (AST, GGT, and LDH) and serum bilirubin and cholesterol concentrations in 6 crossbred Limousine x Angus-Hereford ruminant steer calves (230 kg BW) fed a corn diet containing 205 μmoles of FBs/kg diet for 31 d. The FBs were produced by *F. moniliforme* culture on corn. All of the calves experienced impaired lymphocyte blastogenesis and two of the calves developed mild microscopic liver lesions. These data suggested that FBs were acutely toxic to liver and kidneys of ruminant lambs and, to a lesser extent, ruminant steer calves.

Fumonisins caused an increase liver and kidney toxicity biomarkers in nonruminants, rats and pigs, and ruminants, lambs and steers. However, FBs were at least 14-fold less toxic to steers than lambs suggesting that bovine may absorb less FBs than lambs.

4. Developmental toxicity

Floss (1994) administered 11 μmoles FBs/kg BW/d to 8 d post-mated pregnant

Syrian hamsters by gavage for 3 d resulting in prenatal fetal death. The FBs were produced

by *F. moniliforme* M-1325 on SCM, extracted with water, and filtered. The FB-dosed

hamsters had decreased BW gains and serum bilirubin levels compared to control hamsters.

There were no differences in fetal BW, numbers of prenatal deaths, or resorptions in 8 d post-

mated pregnant Syrian hamsters gavaged with ≤8.3 μmoles FBs/kg BW/d for 3 d. Serum

AST activities between FB_1 treated and control dams were not different indicating that the

fetal deaths were not induced by liver toxicity in the hamster dams. Floss *et al.* (1994),

administered 25 μmoles FB_1 (98% pure)/kg BW/d to 9 d post-mated pregnant Syrian

hamsters by gavage resulting in prenatal fetal death. The FB_1 was produced by *F.*

moniliforme M-1325 on SCM. In a similar experiment, reported by Floss *et al.* (1994), 9 d

post-mated pregnant Syrian hamsters were gavaged with 31 μmoles of FBs/kg BW/d for 2 d

which also resulted in prenatal fetal death. The FBs were produced by *F. moniliforme* M-

1325 on SCM, extracted with water, and filtered. These FB doses that were toxic to fetuses

did not induce clinical maternal intoxication confirming the findings of Floss (1994) that the

fetal deaths were not induced by liver toxicity in hamster dams. Lebepe-Mazur (1993)

administered 84 μmoles FB_1 (80% pure)/kg BW/d to 9 d post-mated pregnant Fisher rats by

gavage for 5 d resulting in suppressed fetal growth and bone development. The FB_1 was

produced by *F. proliferatum* M-5991 in SCM.

Javed *et al.* (1993) injected 1 and 10 d old fertile chicken eggs with 1, 10, or 100

μmoles FB_1 (>95% pure)/egg resulting in embryo toxicity. Mortalities of chicken embryos

from 1 d old FB_1-treated eggs were 50, 70, and 100%, respectively, and from 10 d old FB_1-treated eggs were 30, 60, and 90%, respectively. These data suggested that FB_1 was less toxic to the 10 d old chicken embryos than the 1 d old embryos.

In summary, these data suggested that consumption of FBs was teratogenic to hamsters, rats, and chickens.

5. Cytotoxicity

Incubating FB_1 with mammalian cells *in vitro* can induce cytotoxicity as evaluated by a decrease in cell proliferation or morphologic damage. Yoo *et al.* (1992) incubated rat renal epithelial LLC-PK1 cells with 35 μM FB_1 (>95% pure) for 72-h resulting in an inhibition of LLC-PK1 cell proliferation. The FB_1 was produced by *F. moniliforme* MRC-826 on SCM. After removal from the FB_1 treatment, the LLC-PK1 cells exhibited normal growth kinetics and morphology. Therefore, the cytotoxic effect of FB_1 may have been reversible in these LLC-PK1 cells. Concentrations of ≥70 μM FB_1 were cytotoxic to the LLC-PK1 cells 72-h after exposure. Qureshi and Hagler (1992) incubated chicken peritoneal macrophages (PMs) with 0.69 μM FB_1 (95% pure) for 2-h causing PM cytotoxicity. Morphological damage of the PM cells caused by FB_1 exposure included cytoplasmic blebbing and/or nuclear disintegration. Phagocytic potential of PM cells decreased after 4-h of incubation with 27.7 μM FB_1. There were no increases in FB_1 cytotoxicity caused by the addition of hepatic mixed function oxidase (MFO) enzymes to the incubation medium. Furthermore, the addition of 55 μM FB_1 to the PM culture medium had no effect on the secretion of a cytolytic factor after exposure to lipopolysaccharide. Chatterjee *et al.* (1995) incubated chicken PMs with 8.3 μM FB_1 for 4-h causing nuclear disintegration in the PMs. The FB_1 was produced

by an unidentified fungi that, based on morphology, may have been *F. moniliforme* MRC-826. Fumonisin B_1-damaged macrophages could have caused impaired immune functions in chickens making them susceptible to diseases. However, FBs were not acted on by chicken hepatic MFOs, and FB_1 did not inhibit the secretion of a cytolytic factor by chicken PMs. These data suggested that chickens or turkeys may have had a decreased resistance to bacterial infection after consumption of FBs. Gelderblom *et al.* (1996) incubated male Fisher primary rat hepatocytes with 500 μM FB_1 (98% pure) for 24-h resulting in inhibition of palmitate incorporation into the rat hepatocytes suggesting altered lipid synthesis. The FB_1 induced cytotoxic effects and altered growth responses may have been attributed to fatty acid changes in the major membrane phospholipids and altered fatty acid content of the rat hepatocytes. These data suggested that FB_1 may have caused cytotoxicity in rat epithelial LLC-PK1, chicken PMs, and rat hepatocyte cells.

Comparing the cytotoxic effects of FB_1 and FB_2 in cell cultures may suggest a possible toxicity mechanism based on lipophilicity of these compounds FBs. Cawood *et al.* (1994) incubated primary hepatocytes from male Fisher rats with $[^{14}C]$-FB_1 or $[^{14}C]$-FB_2 (>95% pure) for 48-h resulting in hepatocyte cytotoxicity. The minimum cytotoxic concentrations were 300 μM for $[^{14}C]$-FB_1 and 150 μM for $[^{14}C]$-FB_2. The FBs were produced by *F. moniliforme* MRC-826 on SCM. Cytotoxicity was correlated with the release of LDH from the hepatocytes. The effective dose levels (EDL) [i.e. the lowest level of toxin required to elicit a cytotoxic effect] for binding of FBs to hepatocytes were similar at about 320 μmoles of FB_1 or FB_2/mg protein. There was no indication of metabolism of FB_1 by the rat liver microsomes after incubating them with $\leq$600 μM FB_1. Dombrink-Kurtzman *et al.*

(1994) incubated turkey lymphocytes with FB_1 or FB_2 (both >95% pure) for 72-h inhibiting

cell proliferation. The FBs were produced by *F. proliferatum* in SRM. The IC_{50} for

inhibition of turkey lymphocytes was 1.9 μM FB_1 and 0.6 μM FB_2 suggesting that FB_2 was

over 3-fold more cytotoxic than FB_1 to turkey lymphocyte cells. Cell proliferation was

evaluated by the MTT bioassay in which the tetrazolium salt, MTT, was reduced to MTT

formazan by metabolically active cells. These data suggested that FB_2 exhibited a higher

cytotoxicity and specific binding to primary rat hepatocytes than FB_1. These data are

consistent with the higher lipophilicity of FB_2 than FB_1, which suggests that FB_2 is better at

penetrating cell membranes than FB_1.

The fact that FB_2 was more lipophilic than FB_1 and was more toxic to cell cultures

suggested that the more lipophilic HFBs would be more toxic, on a dietary basis, than their

respective FBs. In addition, the FAs would be expected to be less toxic than their respective

FBs based on the blocked amine group which was thought to play a role in cytotoxicity.

Gelderblom *et al.* (1993) incubated male primary Fisher rat hepatocytes with FB_1, FB_2, FB_3,

HFB_1, HFB_2, FA_1, and FA_2 (all >90% pure) for 48-h resulting in hepatocyte cytotoxicity.

Cytotoxicity was correlated to the release of LDH from the hepatocytes into the culture

medium. Fumonisin B_2 had the highest cytotoxicity on a molar basis to the hepatocyte cells

followed by FB_3 and then by FB_1. The CD_{50} which caused a 50% release of LDH from the

hepatocytes for FB_2 and FB_1 were 1000 and 2000 μM, respectively. The cytotoxicities of

HFB_1 and HFB_2 were greater and the cytotoxicities of FA_1 and FA_2 were less than their

respective parent compounds (FB_1 and FB_2). There was no cytotoxicity observed in these

cells when incubated with the TCA groups. Abbas *et al.* (1993) incubated rat hepatoma

(H4TG), dog kidney (MDCK), and mouse 3T3 fibroblast (3T3) cells with FB_1, FB_2, HFB_1, HFB_2, FA_1, and FA_2 (all >90% pure) for 24-h. The FBs and FAs were produced by *F. moniliforme* MRC-826 on SCM. The IC_{50} values for FB_1 and FB_2 were 18 and 2 μM, respectively, for H4TG cells and 36 and 20 μM, respectively, for MDCK cells. The IC_{50} values for HFB_1 and HFB_2 were 5 and 37 μM, respectively, for H4TG cells, 93 and 20 μM, respectively, for MDCK cells, and 33 and 4.7 μM, respectively, for mouse 3T3 cells. There was no observed toxicity to3T3 cells incubated with ≤70 μM of FB_1 or FB_2. The FAs were not toxic to any of these cell lines at ≤132 μM FAs. These data suggest that FB_2 was more toxic to cell cultures than FB_1 which is consistent with Cawood *et al.* (1994) and Dombrink-Kurtzman *et al.* (1994). The HFBs appear to be equally cytotoxic to their relative FBs when incubated with cell cultures at equal concentrations. Detoxification of FBs may require the removal or blockage of the free amino group which may be needed for its cancer initiating activity. The lack of toxicity of the FAs to rat hepatocytes, H4TG, MDCK, and 3T3 cell lines may be due to their blocked amine group which could play a crucial role in the toxicity of the FBs and HFBs. The FBs had different cytotoxicities to mammalian cell lines. Fumonisin B_2 was more cytotoxic than FB_1 to rat hepatocytes, turkey lymphocytes, H4TG, MDCK, and 3T3 cells. Furthermore, HFB_2 was more cytotoxic than FB_2 in rat hepatocytes and 3T3 cells suggesting increased penetration of cells by HFB_2 than by FB_2. This same pattern of toxicity and lipophilicity existed between HFB_1 and FB_1.

Huang *et al.* (1995) incubated African green monkey kidney (CV-1) cells with 1 μM FB_1 (>98% pure) for 3-h which caused a 2-fold decrease in PKC expression. At 5 μM FB_1, there was a 5-fold AP-1-dependent transcription repression confirming that FB_1 causes PKC

repression in CV-1 cells. The FB_1 caused a 10-fold stimulation of a simple promotor which contained a cyclic AMP response element independent of protein kinase A (PKA). The ability for FB_1 to repress specific PKC isoforms and alter signal transduction pathways may play a role in its toxicity towards CV-1 cells. The ability for FB_1 to decrease PKC expression may suggest a role in its ability to cause cancer.

6. Carcinogenicity

Previous studies have suggested that carcinogens may cause an increase in GGT-altered hepatic foci (AHF) in rats (Goldsworthy et al., 1986; Scherere and Emmelot. 1975). The induction of cancer by chemical compounds can occur by initiation followed by promotion (Emmelot and Scherer 1980; Pitot and Sirica, 1980). A compound that causes initiation or promotion is considered a carcinogen. Gelderblom et al. (1993) fed Male Fisher rats (150 g BW) 1400 µmoles FBs/kg diet for 21 d followed by 0 µmoles FBs/kg diet for 14 d. The rats were dosed with 2-acetylaminofluorene followed by a partial-hepatectomy and fed 0 µmoles FBs/kg diet for 14 d. The rats were sacrificed and liver cancer was observed as determined by the presence of GGT-AHF. The control rats did not develop liver cancer. The FBs were produced by *F. moniliforme* on SCM. No carcinogenicity was observed in rats which consumed 1400 µmoles of FAs, HFBs, or tricarboxylic acid (TCA)/kg diet for 21 d followed by the promoting treatment suggesting that the intact FBs are cancer initiators in male Fisher rats. These data suggested that FB_1 may have been a cancer initiator.

Gelderblom et al. (1988) fed a corn diet containing 10.4 µmoles FB_1/kg diet for 4 wk to male BD-IX rats (150 g BW) that have been initiated for liver cancer with DEN resulting in elevated numbers of GGT-(+) altered hepatic foci (AHF) relative to that of rats which

received only the DEN-initiation treatments. The FB_1 was produced by *F. moniliforme* MRC-826 on SCM. Cancer and toxic effects (decreased BW and hepatitis) were evident in the FB_1 treated rats. Hendrich *et al.* (1993) fed a corn diet containing 69 μmole FB_1/kg diet to DEN-initiated male Fisher rats causing an increase in plasma glutamate-pyruvate transaminase and hyperplastic liver nodules. Lebepe-Mazur *et al.* (1995) fed a corn diet containing 27 μmoles FB_1/kg diet for 6 months to female DEN-initiated Sprague-Dawley rats (6 wk old) resulting in an increased number of placental glutathione S-transferase (+)-AHF. The FB_1 was prepared from *F. proliferatum* M-5991 grown on SCM. These data suggested that FB_1 was a cancer promoter to male and female rats in these experiments.

Voss *et al.* (1993) fed 69 μmoles FB_1 (≥99% pure)/kg diet for 4 wk to non-initiated male and female Sprague-Dawley rats (4 wk old) resulting in liver and kidney cancer in the rats. The FB_1 was produced by *F. moniliforme* MRC-826 on SCM. All rats developed microscopic liver lesions characterized by scattered focal (single cell) hepatocellular necrosis and hepatocellular cytoplasmic vacuolation which appeared more advanced in females. No differences in behavior, food consumption, or BW were observed in any of the rats. Cortical nephrosis was found in male rats fed 21 μmoles FB_1/kg diet and in female rats fed 69 μmoles FB_1/kg diet. Relative kidney weights were decreased in male rats but not female rats fed 69 μmoles FB_1/kg diet compared to controls. Hepatic and renal lesions were observed. Both male and female rats developed liver cancer after they consumed ≥69 μmoles FB_1/kg diet. However, the kidneys of male rats were more sensitive to than female rats to FB_1.

These data suggested that male rats were 3-fold more sensitive than female rats to FB_1 induced kidney cancer but equally sensitive to FB_1 induced liver cancer.

7. Atherosclerotic effects of FBs

Vervet monkeys (1.4 y, 1.76 kg BW) were fed corn containing 2.5 μmoles FBs/kg

BW/d for 4.5 yr which induced indicators of atherosclerosis such as plasma fibrinogen and

blood coagulation factor VII (Fincham et al., 1992). The FBs were produced by F.

moniliforme MRC-826 on SCM. In addition, there was an elevation in plasma low density

lipoprotein-C and apolipoprotein-B in these primates suggesting that symptoms of

atherosclerosis may have been caused by FBs. Smith et al. (1996) fed a corn diet containing

50 μmoles HFB_1/kg BW/d for 7 d to male castrated cross-bred pigs (40 kg BW) resulting in

cardiovascular disease in the pigs. Symptoms of cardiovascular disease caused by HFB_1 in

the pigs included decreases in maximal rate of change of left ventricular pressure, heart rate,

cardiac output, mean aortic pressure, arterial and mixed venous blood O_2 tension, and

systemic O_2 delivery. Other cardiovascular symptoms caused by HFB_1 in the pigs included

increases in mean pulmonary artery pressure, pulmonary vascular resistance, O_2

consumption, and O_2 extraction ratio. These data suggested that low levels of FBs may have

increased the risk of secondary vascular disease in primates. Also, HFB_1 induced left-sided

heart failure in swine.

I. Bioavailability of Fumonisin in Rats

The measure of a compound's bioavailability includes administering the compound in

an animal model and quantification of that compound in excreted material or in tissues.

Bioavailability of FBs in rats is an estimate of the dose exposure these compounds have in

the animals which is affected by absorption, excretion, and accumulation. Absorption of an

oral dose is hindered by the GI tract barrier. Analysis of FBs in biological tissues is difficult. Furthermore, enzymatic degradation of FBs could result in decreased FB recoveries using HPLC-fluorometric analysis. However, there have been no reports of enzymatic degradation of FBs.

Amounts of unmetabolized FB_1 in the urine and feces of rats dosed by gavage with FB_1 were quantified by HPLC fluorometric analysis of the OPA-FB_1 derivative. Shephard et $al.$ (1992a) administered 10.4 μmoles of FB_1/kg BW to fed male BD-IX rats (150 g BW) by gavage and observed 0.4% of the FB_1 dose was excreted in the urine after 48-h. Hopmans et $al.$ (1997) gavaged fed male Fisher rats (9-10 wk old, 160-190 g BW) with 0.69, 6.93. or 69.3 μmoles of FB_1 (70% pure)/kg BW and observed 110, 92, and 98% of the dose, respectively, was excreted in the feces and 7.4, 1.2, and 0.5% of the dose, respectively, was excreted in the urine over 96-h after dosing. The combined data of the three FB_1 levels suggested that FB_1 was excreted nearly completely in the feces (101%) with only a small part in the urine (2.7%) of the rats after 96-h. However, increasing the FB_1 dose decreased the percentage of the total dose that was recovered in the urine suggesting that at a higher FB_1 dose, a lesser percentage of total dose was absorbed or a greater percentage of total dose was excreted though bile of these rats. These data suggested that FB_1 administered by gavage was eliminated in the urine at 7% of the 0.69 μmoles of FB_1/kg BW dose in fed male Fisher rats. However, only 0.4% of the 10.4 μmoles of FB_1/kg BW dose was excreted in the urine of fed male BD-IX rats (Shephard et $al.$, 1992a) and 1.2% of the 6.9 μmoles of FB_1/kg BW dose recovered in the urine of the Hopmans's et $al.$ (1997) male Fisher rats.

Similar to the gavage treatments, biological tissues of rats dosed intraperitoneally were quantified using HPLC-fluorometric analysis. Shephard *et al*. (1992a) administered 10.4 μmoles of FB_1/kg BW to fed male BD-IX rats (150 g BW) intraperitoneally. The FB_1 was rapidly absorbed into the blood reaching a maximum concentration of 12 nmoles/ml in plasma of the rats after 20 min. The FB_1 had a relatively short plasma half life of 18 min after dosing. Only 16% of the dose was eliminated unmetabolized in the urine of the rats 24-h after dosing. These data suggested that urinary excretion was a major route for elimination of FB_1 by rats.

Scintillation counting of radioactive [^{14}C]-FB allows for improved detection of FBs in biological tissues over HPLC-fluorescence methods. Fumonisin B_1 has been radioactively labeled when [^{14}C]-acetate or [^{14}C]-methionine were added to cultures of *F. moniliforme* or *F. proliferatum* in LM. Norred *et al*. (1993) dosed fasted male Sprague-Dawley rats (150-200 g BW) by gavage with 1.4 μmoles of [^{14}C]-FB_1/rat. The rats excreted 80% of the [^{14}C]-dose in the feces 48-h after dosing. Within 96-h after dosing, 2-3% of the [^{14}C]-dose was excreted in the urine, while the liver accumulated a maximum of 0.5% of the [^{14}C]-dose 4-h after dosing. The blood and kidneys retained 0.2 and 0.1% of the [^{14}C]-dose 96-h after dosing. Shephard *et al*. (1992b) dosed fed male BD-IX rats (6 wk old, 150 g BW) by gavage with 10.4 μmoles of [^{14}C]-FB_1 (96% pure)/kg BW. All of the [^{14}C] was recovered in the feces and trace amounts were found in the urine, liver, kidneys, and blood after 24-h. Shephard *et al*. (1994a) cannulated and dosed by gavage fed male Wistar rats (6-8 months old, 350-400 g BW) with 10.4 μmoles of [^{14}C]-FB_1 (93% pure)/kg BW. The rats excreted 0.2% of the [^{14}C] dose in the bile within 24-h. Comparing fasted Sprague-Dawley rats gavaged with 6.9

μmoles of [^{14}C]-FB$_1$/kg BW (Norred *et al.*, 1993) to fed Fisher rats gavaged with 6.9 μmoles

of FB$_1$/kg BW (Hopmans *et al.*, 1997), the fasted rats absorbed and excreted in the urine 2-

fold more FB$_1$ than fed rats. However, biliary excretion of FB$_1$ may have been responsible

for excretion of 0.2% of the dose in both studies. In addition, scintillation counting of [^{14}C]-

FB$_1$ may have quantified metabolized and unmetabolized FB$_1$ (Norred *et al.*, 1993), while the

fluorometric analysis of FB$_1$ only quantified unmetabolized FB$_1$ (Hopmans *et al.*, 1996).

Norred *et al.* (1993) administered 6.2 nmoles of [^{14}C]-FB$_1$/rat to fasted male Sprague-

Dawley rats (150-200 g BW) by iv. The [^{14}C]-FB$_1$ dose was equivalent to 0.03 μmoles of

[^{14}C]-FB$_1$/kg BW for a 200 g rat and produced 10,000 decays per min (dpm). The rats

excreted 35 and 10% of the [^{14}C]-dose in the feces and urine, respectively, and 30, 10, and

2% of the [^{14}C]-dose was recovered in the liver, kidneys, and GI tract, respectively, after 96-

h. Shephard *et al.* (1992b) dosed fed male BD-IX rats (6 wk old, 150 g BW)

intraperitoneally with 10.4 μmoles of [^{14}C]-FB$_1$ (96% pure)/kg BW. The [^{14}C]-FB$_1$ dose

produced 100,000 dpm/rat. The rats excreted 66% of the [^{14}C] in the feces and 32% in the

urine 24-h after dosing. Trace amounts of [^{14}C] were recovered in the liver, kidneys, and

blood 24-h after dosing. Shephard *et al.* (1994a) dosed bile duct cannulated male Wistar fed

rats (6-8 months old, 350-400 g BW) intraperitoneally with 10.4 μmoles of [^{14}C]-FB$_1$ (93%

pure)/kg BW. The rats dosed intraperitoneally eliminated 67% of the [^{14}C] dose in the bile

within 24-h after dosing. These data suggested biliary excretion was a major route of

elimination of [^{14}C]-FB$_1$ dosed in these rats. Over half of the absorbed dose of FB$_1$ was

excreted in the bile indicating that FB$_1$ dosed by gavage to rats may have been absorbed by

the GI tract to a greater extent than previously reported.

The bioavailability of compounds similar to FB_1, such as HFB_1 and FB_1-FRU, in rats has recently been reported. Amounts of unmetabolized HFB_1 and FB_1-FRU in urine and feces of fed rats were hydrolyzed and quantified for their OPA-HFB_1 derivatives by HPLC fluorometry. Hopmans *et al.* (1997) gavaged male Fisher fed rats (9-10 wk old, 160-190 g BW) with 0.69, 6.93, or 69.3 μmoles HFB_1 (70% pure)/kg BW and observed 15.1, 1.3, and 0.2% of the total dose, respectively, was excreted in the urine. Similarly, fed rats gavaged with 0.69 or 6.93 μmoles FB_1-FRU/kg BW excreted 9.8 and 0.8% of the total dose in the urine, respectively. Increasing the HFB_1 or FB_1-FRU dose decreased the percentage of the doses that were recovered in the urine. Similar to the FB_1 treatments by Hopmans *et al.* (1997), increasing the HFB_1 or FB_1-FRU dose decreased the percentage of the total dose that was recovered in the urine suggesting that at a higher HFB_1 or FB_1-FRU dose, a lesser percentage of total dose was absorbed or a greater percentage of total dose was excreted though bile of these rats. These data suggested that absorption of 0.69 μmoles of HFB_1 or FB_1-FRU occurred in these rats dosed by gavage and was eliminated in the urine at 15.1 and 9.8% of the total dose, respectively. Biliary excretion may have been responsible for some of the elimination of these compounds from the rats as suggested by biliary excretion of [^{14}C]-FB_1 by Shephard *et al.* (1994a). If biliary excretion of HFB_1 and FB_1-FRU occurred to a lesser extent than FB_1, then this might explain why higher percentages of HFB_1 and FB_1-FRU relative to FB_1 were eliminated in the urine of these rats.

Fumonisin B_1, HFB_1, and FB_1-FRU are absorbed by the GI tract of rats. With respect to FB_1, less than 5% of a gavaged dose is absorbed and excreted in the urine or accumulated in the tissues. However, biliary excretion of FB_1 is only slightly occurring suggesting that

the absorption of FB₁ is only slightly greater than the amount excreted in the urine. Gavaging

unfed rats appears to increase the absorption of FB₁. However, a study of similar rats and at

the same doses which could measure this has not been reported.

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CHAPTER 3. FUMONISIN B₁ PRODUCTION BY *FUSARIUM PROLIFERATUM* STRAIN M5991 IN A MODIFIED MYRO LIQUID MEDIUM

A paper published in the Journal of Natural Toxins[1]

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Abstract

Fusarium proliferatum strain M5991 cultures were grown in shake flasks containing

modified Myro (MM) medium ($MgSO_4$ reduced to 0.5 g/L) plus 0. 0.25. 0.50. 0.75. 1.00. or

1.25% (v/v) hot-water corn-hull-extract (CHE) for 69 days. After 4 days of incubation. shake

flask liquid cultures with 0.75, 1.00, and 1.25% (v/v) CHE showed a reduction in pH from

6.0 to 2.6 and consumed sucrose at >6.3 g/L/d. After 69 days of incubation, the same shake

flask cultures produced over 7.8 g/L cell mass and over 990 mg/L fumonisin B₁ (FB₁). A

minimum CHE level of 0.75% was recommended for enhanced FB₁ production by *F.*

proliferatum strain M5991. During three serial (10, 12, and 12-L) batch fermentations in

MM medium + 1.00% (v/v) CHE (first batch only), *F. proliferatum* strain M5991 produced

FB₁ concentrations of 619, 659, and 375 mg/L after 35, 47, and 52 days of incubation,

respectively. By analysis, a total yield of 20 g FB₁ was obtained from three serial batch

fermentations.

Key words: Liquid Fermentation, Corn Hull Extract, Maize Pathogen, FB₁, Fungus

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Introduction

The fumonisins (FBs) are a family of mycotoxins including FB_1, FB_2, FB_3, and FB_4 (Wilkes *et al.*, 1995; Beier *et al.*, 1995) with FB_1 the most predominant. Fumonisins are produced by the maize pathogens, *Fusarium proliferatum* and *Fusarium moniliforme*. The fumonisins cause various toxic symptoms in different animal species.

Leucoencephalomalacia (LEM) was induced in a male horse after 20 doses containing 1 to 4 mg of 95% pure FB_1/kg diet (total dose 8.4 g FB_1) for 29 days (Kellerman *et al.*, 1990). Five ponies developed LEM after feeding on diets containing 8 mg FB_1/kg diet for 180 days (Wilson *et. al.*, 1992). Pulmonary edema was induced in weanling pigs fed 92 mg FB_1/kg diet (Osweiler *et al.*, 1992) and in 16-24 kg pigs fed corn screening diets containing 155 mg FB_1/kg diet for 7 days (Colvin and Harison 1992). Embryopathenicity was induced in chicken embryos when eggs were inoculated with 72 ng FB_1 per egg (Javed *et al.*, 1993). Developmental toxicity was observed in hamsters dosed by gavage with >6 mg FB_1/kg diet (Floss *et al.*, 1994). Liver cancer occurred in rats fed a 50 mg pure FB_1/kg diet for 26 months (Gelderblom *et al.*, 1991). Renal lesions developed in male rats fed 15 mg of a 99% pure FB_1/kg diet for 4 weeks (Voss *et. al.*, 1993). In contrast, FBs appeared to be only slightly toxic to calves fed 148 mg total FBs/kg diet (Osweiler *et al.*, 1992). The effects of FBs on human health are not known, but human esophageal cancer may be associated with FB consumption (Chu and Li, 1994). FB_1 has been declared a class 2B carcinogen (IARC, 1993).

The purified FB_1 used in most of the previous studies cited has been isolated from solid corn culture fermentations of either *F. moniliforme* or *F. proliferatum*. Liquid-culture

fermentations alleviate some purification problems caused by impurities derived from solid-state fermentations of *F. proliferatum* on corn or rice which co-purify with FB_1 (Miller *et al.*. 1994).

Preliminary data illustrated that growth and fumonisin production of *F. proliferatum* strain M5991 were enhanced by the addition of 10 ml Corn-hull-extract (CHE) to 1-L modified Myro (MM) medium. In this paper, we report significantly enhanced cell growth and FB_1 production by *F. proliferatum* strain M5991 through the addition of a hot-water CHE in shake flask cultures and in a 10-L repeated batch fermentation.

Materials and Methods

Liquid media

Myro (M) medium consisted of 1.0 g $(NH_4)_2HPO_4$ (Fisher Scientific. Fair Lawn, N. J.). 3.0 g KH_2PO_4 (Fisher), 2.0 g $MgSO_4 \cdot 7H_2O$ (Baker Chemical Co., Phillipsburg, N.J.), 5.0 g NaCl (Fisher), 40 g sucrose (Fisher), and 10 g glycerin (Fisher) in 1-L distilled-water (dH_2O); pH 5.9 (Chelkowski, 1989). Modified Myro (MM) medium consisted of M medium reduced to 0.5 g/L $MgSO_4 \cdot 7H_2O$. Reduced sucrose modified Myro (RSMM) medium consisted of MM medium reduced to 20 g/L sucrose.

Corn-hull extract (CHE) was prepared by autoclaving 10 g of corn hulls (crude corn fiber, Penford Product Corn, Cedar Rapids, IA) with 100 ml of dH_2O for one hour 121°C followed by centrifugation at 13,776 x g for 10 minutes. The clarified CHE was made to 100 ml volume using dH_2O, sterilized at 121°C for 20 minutes, and stored at ambient temperature until needed.

Fungal culture

Lyophilized fungal spores of *F. proliferatum* strain M5991, a predominant FB_1 fumonisin-producing isolate, were obtained from Dr. Paul Nelson (Pennsylvania State University, University Park, PA). The fungal spores were rehydrated in capped 1-L baffled Erlenmeyer flasks containing 500 ml of sterile RSMM medium. The rehydration flasks were incubated for 7 days at ambient temperature on a New Brunswick G10 rotary shaker (New Brunswick Scientific Co., Inc. Edison, NJ) at 220 rpm (2.5 cm throw) and the cells were collected by centrifugation at 8,671 x *g* for 10 minutes . The fungal cells were stored as followed using the method of Windels *et al.* (1988). The concentrated fungal cells were resuspended in 100 ml of sterile 10% skim milk broth (Shoppers Value Instant nonfat dry milk, Preferred Products Inc., Eden Prairie, MN). Aliquots (0.5 mL) containing the resuspended fungal cells were added drop-wise to 19 x 150 mm screw-capped tubes containing 15 g of sterile silica gel beads (grade 40, 6-12 mesh) (Aldrich Chemical Co., Milwaukee, WI), shaken, and stored at 4°C until needed.

Inoculum was prepared by transferring 10 to 20 of the fungal-containing silica beads into a 1-L baffled Erlenmeyer flask containing 500 ml of sterile MM medium plus 1% (v/v) CHE and incubating for 4 days at ambient temperature with shaking at 220 rpm. These conditions yielded 5×10^7 CFU/mL in logarithmic phase.

Shake flask cultures

Cultures (5 ml inoculum) were grown in 1-L Erlenmeyer flasks containing 500 ml of MM medium plus 0.00, 0.25, 0.50, 0.75, 1.00, or 1.25% (v/v) CHE. Incubation was at ambient temperature with shaking at 220 rpm (2.5 cm throw) for 69 days. Samples (5 mL)

were removed weekly, filtered through a 0.45 μm (25 mm diameter) filter (Micron

Separations Inc., Westboro, MA), and stored at -20°C until analyzed. Cultures were grown in

triplicate, and 10 samples from each culture were obtained.

Fermentor conditions

Batch fermentation 1

The fermentor vessel (15-L New Brunswick microferm fermentor vessel, New

Brunswick Scientific Co., Inc. Edison, NJ), containing sterilized MM medium (9.8-L) and

CHE (0.1-L) was inoculated with the previously described inoculum (0.1-L) and maintained

at 28°C with 220 rpm agitation. Filter sterilized (0.2 μm) air was pumped into the culture

medium at 2.1 vvm. Foam was controlled by an automatic antifoam controller with antifoam

FM (Hodag Corp., Skokie, IL). Samples were taken every 12 hours for 3.5 days and then

weekly throughout the fermentation. After 35 days of incubation, the agitation was

terminated for 24 hours and the cell mass allowed to settle. Culture filtrate (7-L) was

pumped out via a "J" tube leaving about 2-L of residual cell mass and culture filtrate in the

fermentor vessel. The harvested culture filtrate was stored at 5°C until analyzed for FB_1.

Batch fermentation 2

The fermentor, containing residual cell mass and culture filtrate, was refilled

aseptically to a total 12-L with sterile MM medium. Samples were taken weekly throughout

the fermentation. After 47 days of incubation, the agitation was terminated for 12 hours and

the cell mass allowed to settle. Culture filtrate (7-L) was pumped out via a "J" tube leaving

about 4-L of residual cell mass and culture filtrate in the fermentor vessel. The harvested

culture filtrate was stored at 5°C until analyzed for FB_1.

Batch fermentation 3

The fermentation proceeded as described in batch fermentation 2. After 52 days of

incubation, all of the residual cell mass and culture filtrate (11-L) were collected from the

fermentor vessel and stored at 5°C until analyzed for FB_1.

Each serial batch fermentation lost about 0.9-L dH_2O due to evaporation and an

additional 0.1-L of culture filtrate was removed during sampling.

Fungal growth analysis

pH

The pH of liquid culture samples from shake flasks and fermentation batches 2 and 3

were measured using a Corning Model 12 research pH meter. The pH from batch

fermentation 1 medium was measured in the fermentor vessel using an Ingold sterilizable pH

electrode.

Reducing sugars

Total reducing sugars were quantified by Somogyi-Nelson method (Somogyi, 1945;

Nelson 1944). Samples containing < 120 μg reducing sugar were prepared by transferring 1

ml of sample into 10 ml volumetric flasks containing 5 ml of HCl (6 N), mixing, and

allowing to stand at room temperature for 24 hours. The pH was adjusted to 7 with NaOH

(10 N), filled to volume (10 mL) with dH_2O then mixed. The samples were analyzed for

absorbance at 520 nm and compared to a hydrolyzed sucrose standard curve.

Cell density

Cell densities were determined by filtering measured volumes (2-5 mL) of liquid

culture samples through a preweighed 0.45 μm (25 mm diameter) filter (Micron Separations

Inc., Westboro, MA). Cells were dried at 60°C for 24 hours, equilibrated to room

temperature, then weighed.

Fumonisin analysis

Fumonisin B_1 concentrations were measured according to the procedure of Hopmans

and Murphy (1993). Aliquots (100 μl) containing 0.45 μm filtered fermentation medium

were derivatized for 10 minutes with 100 μl o-phthaldialdehyde (OPA) solution (5 mg OPA,

5 ml acetonitrile, and 10 μl 2-mercaptoethanol), 100 μl buffer (0.05 M K_2HPO_4 pH adjusted

to 8.3 with 0.05 M KH_2PO_4), and 100 μl dH_2O. The derivatized samples were diluted with

100 μl of dH_2O and injected (20 μl) into a high performance liquid chromatography (HPLC),

(Beckman Instruments, Fullerton CA) with analytical reverse phase C_{18} column (Perkin and

Elmer Corp., Norwalk, CT) (3mm ID, and 4.6 cm length). The mobile phase was 40%

acetonitrile and 60% 0.05 M KH_2PO_4 (pH adjusted to 3.3 with concentrated phosphoric acid)

at a flow rate of 1.5 ml per minute. The FB_1 fluorescent derivatives were quantified using a

Turner fluorometer (Corning 7-60 primary filter and Wratten 2a secondary filter, 15 μl flow

cell) and compared against a FB_1 standard generously donated by P.G. Thiel of the Research

Institute for Nutritional Diseases, South African Medical Research Council, South Africa.

CHE analysis

A 10-ml preweighed sample of CHE was evaporated in a weighed crucible over a

Bunsen burner flame followed by heating in a muffled furnace at 1000°C for 8 hours. The

ashed sample was analyzed for calcium (Ca), potassium (K), magnesium (Mg), sodium (Na),

phosphorous (P), and zinc (Zn) by inductively coupled plasma-atomic emission (ICP-AE)

spectroscopy (Fisons, 3410 ICP with minitorch, Beverly MA.) (Analytical Services Division

of Ames Laboratory, United States Dept. of Energy, Iowa State University, Ames IA).

Statistical analysis

The experimental design for the CHE study consisted of six treatments: 0.00, 0.25,

0.50, 0.75, 1.00, and 1.25% (v/v) CHE. Each treatment was replicated in three different

flasks. Data were analyzed using ANOVA least significant difference (LSD) Test for

variable: Y (model y = treatment, n = 3) on SAS system v. 6.07 (Cary, NC) (p < 0.05).

Results and Discussion

Corn hull extract

The enhancement of cell growth and FB_1 production by *F. proliferatum* strain M5991

by the addition of CHE was discovered while investigating surface attachment abilities of the

mold to plastic composite supports (40% of either oat, soy, barley, or corn hulls; 10% yeast

extract; and 50% polypropylene; Demirci *et al.* [1993]). The CHE used in this study was

made from 10 g wet-milled corn hulls per 0.1-L of dH_2O and contained 47.0 g dissolved

solids/L (3.9 g ash/L). The ICP-AE-analyzed CHE contained: Ca (0.02 g/L), K (1 g/L), Mg

(0.3 g/L), Na (0.06 g/L), P (0.71 g/L), and Zn (0.01 g/L).

Shake flask liquid culture study

The culture pH reduction could not be attributed to lactic, acetic, or propionic acid

production as determined by HPLC (Demirci *et al.*, 1993). However, there were two

unidentified chromatographic peaks observed by refractive index detection. The pH of the

culture medium decreased quickest in the treatments containing the highest levels of CHE

(Fig. 1). The shake flask cultures containing the two highest levels of CHE (1.00 and 1.25%) consumed all of the sucrose in the shortest incubation time (32 d) (Fig. 2A). The cell mass production plateaued at 32 days incubation for the 0.75, 1.00, and 1.25% CHE media (Fig. 2B). The FB_1 concentration plateaued after 32 days of incubation in the 1.00 and 1.25% CHE media, after 48 days in the 0.50 and 0.75% CHE media, and was still increasing after 69 days of incubation in the 0.00 and 0.25% CHE media (Fig. 2C). Negligible amounts of FB_2 and FB_3 were detected in media of *F. proliferatum* M5991.

The rate of pH change for all treatments was evaluated between 0 and 2 days because the pH of the 1.25% CHE treatment plateaued after 2 days of incubation (Fig. 1). The rate of reducing sugar and cell density change were evaluated between 0 and 4 days of incubation and represented initial rates (Fig. 2A,B). Rates of pH drop after 2 days of incubation and rates of cell mass production after 4 days of incubation with *F. proliferatum* strain M5991 were significantly greater in the culture media containing 0.50, 0.75, 1.00, and 1.25% CHE than the culture medium containing no CHE ($p < 0.05$) (Fig.s 1 and 2B). The rate of reducing sugar consumption after 4 days of incubation was significantly greater in the culture media containing 0.75, 1.00, and 1.25% CHE than the culture medium containing no CHE ($p < 0.05$) (Fig. 2A).

The 21 days incubation time, in which reducing sugar, cell density, and FB_1 concentrations were evaluated, represented the incubation time in which the FB_1 culture medium containing 1.25% CHE plateaued (Fig. 2A-C). The reducing sugar, cell density, and FB_1 concentrations were significantly greater in the culture media containing 0.50, 0.75, 1.00, and 1.25% CHE than the culture medium containing no CHE after 21 days of incubation ($p <$

0.05). The cell density and FB$_1$ concentrations evaluated after 69 days of incubation

represented the final experimental values (Fig. 2B,C). The cell density and FB$_1$

concentrations were significantly greater in the culture media containing 0.50, 0.75, 1.00, and

1.25% CHE than the culture medium containing no CHE after 69 days of incubation (p <

0.05). However, FB$_1$ concentrations were not significantly different from the culture media

containing 0.75, 1.00, or 1.25% CHE after 69 days of incubation. These latter CHE

concentrations suggest that the minimum concentration of CHE which was required for

optimum production of FB$_1$ by *F. proliferatum* is 0.75%.

These data are consistent with those of Blackwell *et al.* (1994), who found that, under

similar conditions, a higher inoculum of *F. moniliforme* strain NRRL 13616 resulted in

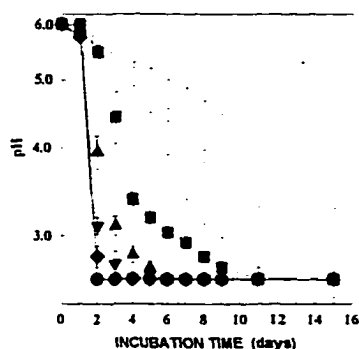increased amounts of total fumonisins produced.

Figure 1. pH response from *Fusarium proliferatum* grown in shake flask cultures. Shake flask cultures of *F. proliferatum* strain M5991 inoculated into modified Myro medium containing 0.00 (O), 0.25 (■), 0.50 (▲), 0.75 (▼), 1.00 (◆), or 1.25 (●) % (v/v) corn-hull-extract. Flasks were incubated at 220 rpm and ambient temperature. Data points represent means. Error bars represent standard deviations (n = 3).
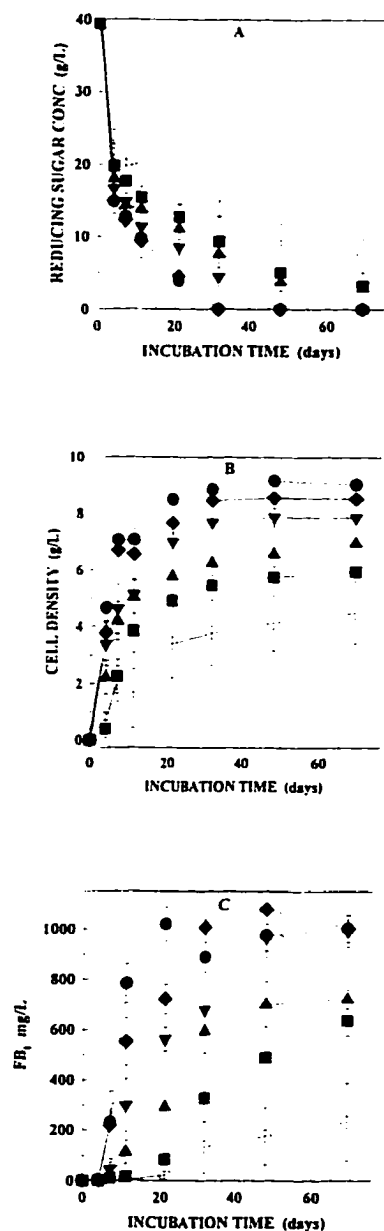
Figure 2. Growth characteristics of *Fusarium proliferatum* in shake flask cultures. (A) Reducing sugar concentration (g/L), (B) cell density (g/L), and (C) FB_1 concentration (mg/L) from shake flask cultures of *F. proliferatum* strain M5991 inoculated into modified Myro medium containing 0.00 (O), 0.25 (■), 0.50 (▲), 0.75 (▼), 1.00 (♦), or 1.25 (●) % (v/v) corn-hull-extract. Flasks were incubated for 69 d at 220 rpm and ambient temperature. Data points represent means. Error bars represent standard deviations (n = 3).

Fermentation study

Batch fermentations produced 25-L of spent fumonisin culture medium in 136 days and yielded 20.4 g of crude FB_1. Changes in pH, reducing sugars. cell mass. and FB_1 were continuously monitored. The pH drop to 2.9 after 2 days (Fig. 3) of incubation during each batch fermentation was similar to the pH drop observed in the shake flask cultures.

After compensating for evaporation and subtracting initial starting concentrations (Fig. 4-C), the repeated serial-batch-fermentations yielded a maximum FB_1 production of 619, 659, and 375 mg/L and a maximum cell mass production of 8.3. 4.4, and 3.3 g/L (dry weight), respectively. The best production of FB_1 and cell mass was achieved during the first fermentation. In addition, the first batch fermentation may have been terminated too early because FB_1 production and sucrose consumption were still occurring at the time of harvest. If the first batch fermentation were to continue to 48 days, the maximum yield of FB_1 could be assumed to reach over 800 mg/L (Fig. 4A). This assumption could lead to the logical conclusion that the yield of FB_1 decreases during the fermentation because the cells were not as healthy during the second and third batches as they were during the first batch. Another factor that could lead to these results is the fact that CHE was only added to the first batch fermentation.

Foaming was apparently not a problem during the first (10-L) batch fermentation; therefore, the volumes of the second and third batch fermentations were increased to 12-L. Because there is cell mass and culture filtrate carryover, the second and third serial batch fermentations were calculated to have initial FB_1 concentrations of 113 and 349 mg/L. respectively. However, the respective FB_1 concentrations were found to be 310 and 320

mg/L. The high measured level of FB$_1$ at 0 hour, in the second batch fermentation, was probably the result of a sampling error and was omitted from Fig. 4B.

Miller *et al.*, (1994) reported total fumonisin (FB$_1$ + FB$_2$) levels of almost 300 mg/L. cell masses of 5.35 g/L, and consumption of all but about 10 g of sucrose after 10 days of fermentation in a similar fermentation system with *F. moniliforme*. In our fermentation with *F. proliferatum* strain M5991, a predominant FB$_1$ producer, FB$_1$ levels (200 mg/L) and cell mass (2 g/L) achieved from our first batch fermentation (Fig. 4A), after 10 days of fermentation, were less than those reported by Miller *et al.*, (1994). However, *F. proliferatum* M5991 is preferred for FB$_1$ purification in our hands since negligible levels of FB$_2$ and FB$_3$ were produced. The cell mass obtained after 10 days of fermentation in the first repeated batch fermentation was about half of that obtained during Miller's fermentation but our cell mass continued to increase throughout 35 days of fermentation.
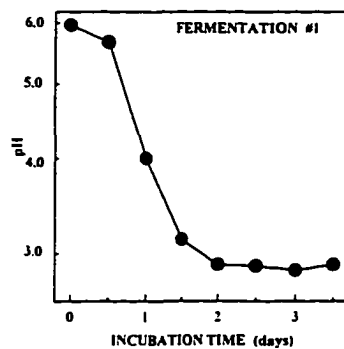

Figure 3. pH response for *Fusarium proliferatum* from fermentation 1 of the repeated batch fermentation.
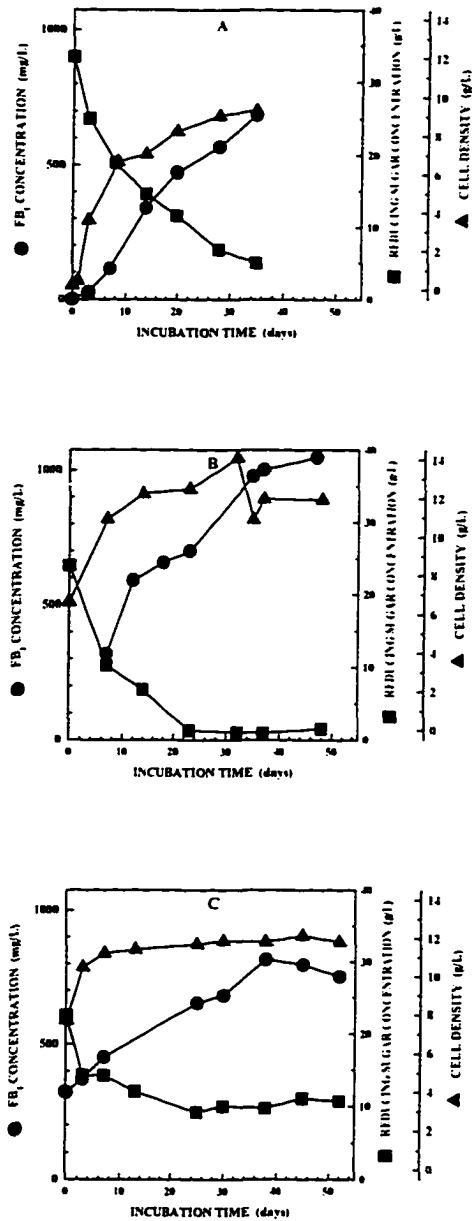
Figure 4. Repeated batch fermentation 1 (A), fermentation 2 (B), and fermentation 3 (C), quantified for fumonisin production (mg/L) (●), reducing sugar consumption (g/L) (■), and changes in dry weight cell-mass (g/L) (▲) for *Fusarium proliferatum* strain M5991. Batch fermentation 1 contained 10-L of Modified Myro medium plus 1% (v/v) corn-hull-extract. Batch fermentations 2 and 3 contained 12-L of Modified Myro medium. These data were measured prior to compensating for evaporation.

Conclusions

Growth and FB$_1$ production by *F. proliferatum* strain M5991 were about the same

with 0.75, 1.00, and 1.25% (v/v) CHE additions in MM medium. Therefore, we recommend

that 0.75% CHE be added to the MM medium for the production of FB$_1$ by *F. proliferatum*

M5991. Repeated batch fermentation has the advantages over non-repeated batch

fermentations by decreasing the down time that would be needed for cleaning, reinoculating,

and initial growth of the culture.

Acknowledgments

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CHAPTER 4. PURIFICATION OF FUMONISIN B₁ FROM LIQUID CULTURES OF FUSARIUM PROLIFERATUM

A paper published in the Journal of Agriculture and Food Chemistry[1]

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Abstract

Fumonisin B₁ (FB₁) was purified from liquid culture medium by adsorption, partition, and ion exchange liquid chromatography. Liquid-culture filtrates of *Fusarium proliferatum* strain M5991 grown in modified-Myro medium and containing ≤ 750 mg FB₁/L were eluted from XAD-16, C₈, DEAE-Sepharose, and C₁₈ resulting in 96-100% pure FB₁ with a 37% recovery.

Introduction

Fumonisins (FBs) are a family of mycotoxins (FB₁, FB₂, FB₃, and FB₄) which 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 lab, we have only obtained 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 $(NH_4)_2HPO_4$, 3.0 g KH_2PO_4, 0.5 g $MgSO_4•7H_2O$, 5.0 g

NaCl, 40 g sucrose, and 10 g glycerin in 1-L 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 one hour at 121°C

followed by centrifugation at 13,776 x g for 10 minutes. The clarified CHE was made to 100

mL volume using dH_2O, sterilized at 121°C for 20 minutes, 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 #1 filter (Whatman

Laboratory Division, Springfield Mill, Maidstone, Kent) followed by 0.8 and 0.45 μm MSi

filters (4.5 cm dia, 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, Ave. Pore Dia.: 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 x 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, New York) at 50°C and filtered through a 0.45

μm MSi filter. The FB-containing filtrates were adjusted to a pH of ≤3.3 using 6 M

hydrochloric acid (HCl).

C₈ 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 FB_1 and introduced onto the

column via 1-3 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 x 1.5 cm tubes (10 mL of eluent/tube)

using a fraction collector (FRAC-100, Pharmacia Fine Chemicals, Piscataway, New Jersey).

The eluents were monitored for FB_1 by thin layer chromatography (TLC), concentrated by RE to

10 mL, filtered through a 0.45 μm MSi filter, and pH adjusted to 6.5 using 10 N sodium

hydroxide (NaOH). The remaining FBs and other contaminants were eluted from the column

with 100 mL of ACN. The column was regenerated with ≥250 mL 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 d 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 x 2

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 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, filtered through a 0.45 µm MSi filter. and pH 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 semi-preparatory C_{18} high performance liquid chromatography (HPLC) column (AM 323-5, Size: 25 x 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 x 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 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

MqH_2O, and lyophilized in a freeze dryer (18 port, Labconco Corp., Kansas City, MO) for

>12 hours.

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 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%.

Table 1. Purity data of fumonisin B_1 containing samples. Fumonisin B_1 (FB_1) mass (mg), recovery (%), and purity (%) obtained from liquid cultures of *Fusarium proliferatum* strain M5991 by XAD-16, C_8, DEAE-Sepharose, and C_{18} liquid chromatography.

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

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

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. These figures show

the lack of any OPA derivatizable contaminants in this sample purified by 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) (Fig. 3).

We were unable to obtain pure FB_1 from solid corn, solid rice, or liquid-Myro media

using Miller's (et al., 1994) silica or Meredith's (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.

Conclusions

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 FB_1 were not altered by the addition of CHE to the liquid Myro medium.
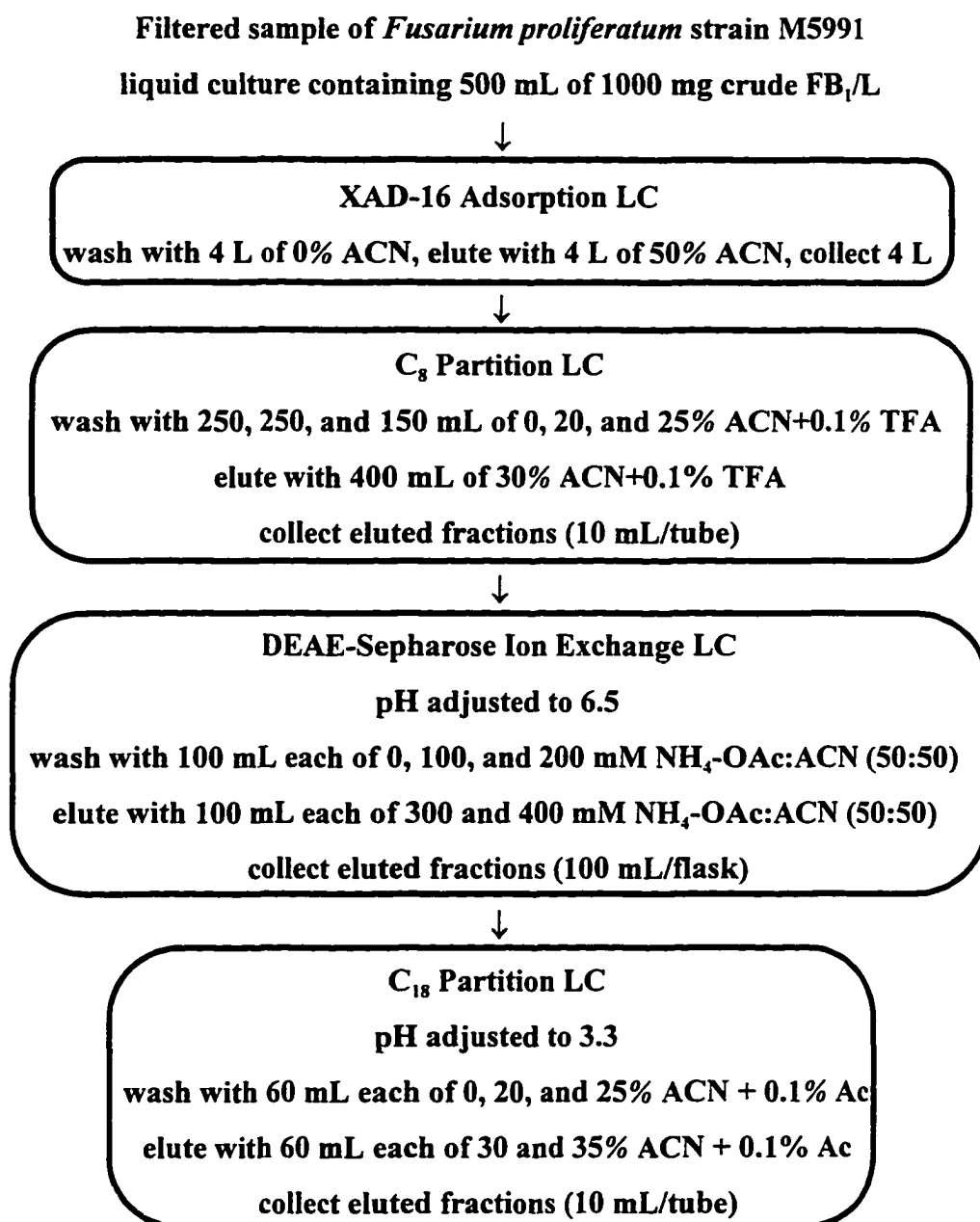
Filtered sample of *Fusarium proliferatum* strain M5991

liquid culture containing 500 mL of 1000 mg crude FB_1/L

↓

XAD-16 Adsorption LC

wash with 4 L of 0% ACN, elute with 4 L of 50% ACN, collect 4 L

↓

C_8 Partition LC

wash with 250, 250, and 150 mL of 0, 20, and 25% ACN+0.1% TFA

elute with 400 mL of 30% ACN+0.1% TFA

collect eluted fractions (10 mL/tube)

↓

DEAE-Sepharose Ion Exchange LC

pH adjusted to 6.5

wash with 100 mL each of 0, 100, and 200 mM NH_4-OAc:ACN (50:50)

elute with 100 mL each of 300 and 400 mM NH_4-OAc:ACN (50:50)

collect eluted fractions (100 mL/flask)

↓

C_{18} Partition LC

pH adjusted to 3.3

wash with 60 mL each of 0, 20, and 25% ACN + 0.1% Ac

elute with 60 mL each of 30 and 35% ACN + 0.1% Ac

collect eluted fractions (10 mL/tube)

Figure 1. Purification procedure for fumonisin B_1 (FB_1).
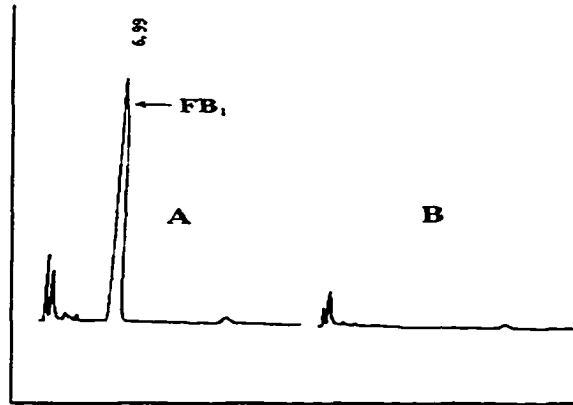
Figure 2. HPLC-OPA chromatogram of fumonisin B_1. Fumonisin B_1 containing sample 84 (4.6 mg fumonisin B_1/L) (A) and a water blank (B).
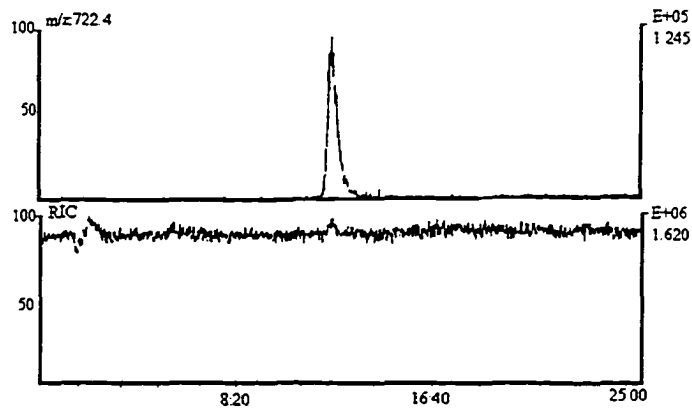

Figure 3. HPLC-MS Chromatogram of fumonisin B_1. Analysis of underivatized fumonisin B_1 in sample 84 analyzed by Dr. R. Plattner using electrospray interface-MS detection (Mode: ESI +Q1MS LMR UP LR; Peak: 1000.00 mmu; Masses scanned: 250 - 950 m/z).

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CHAPTER 5. EXCRETION OF ^{14}C-FUMONISIN B$_1$, ^{14}C-HYDROLYZED FUMONISIN B$_1$, AND ^{14}C-FUMONISIN B$_1$-FRUCTOSE BY RATS

A paper to be submitted to the Journal of Agriculture and Food Chemistry

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Abstract

[^{14}C]-Fumonisin B$_1$ (FB$_1$) was produced by *Fusarium proliferatum* M-5991 in

modified Myro liquid medium (LM) and purified to >95% purity with a specific activity of

1.7 mCi/mmole. Nine male and 9 female F344/N rats were dosed by gavage with 0.69

μmole [^{14}C]-FB$_1$ or [^{14}C]-HFB$_1$ or [^{14}C]-FB$_1$-FRU/kg BW. Urinary excretion of [^{14}C]-HFB$_1$

by female rats was significantly greater than male rats at 17.3 and 12.8% of the dose,

respectively. Urinary excretion by male and female rats combined was 4.4 and 0.5% of the

[^{14}C]-FB$_1$-FRU and [^{14}C]-FB$_1$ dose, respectively. In a biliary excretion study, 9 Sprague-

Dawley female rats were dosed by gavage with 0.69 μmole [^{14}C]-FB$_1$ or [^{14}C]-HFB$_1$ or [^{14}C]-

FB$_1$-FRU/kg BW. There was no difference in the biliary excretion of the 3 FB compounds in

these rats with 1.35% of the dose being excreted in the bile 4 hr after dosing. These data

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suggested that HFB_1 was absorbed to a greater extent than FB_1 in these rats. In a comparison

of HFB_1 and FB_1, both were equally toxic but, on a dietary basis, HFB_1 was absorbed to a

greater extent in rats suggesting that HFB_1 was less toxic at the cellular level. The

detoxification of FB_1 by reaction with fructose was not explained by the absorption of FB_1-

FRU which was greater than FB_1 in rats.

Introduction

The fumonisins (FBs) are a family of mycotoxins including FB_1, FB_2, FB_3, and FB_4

(Gelderblom *et al.*, 1988; Cawood *et al.*, 1991) with FB_1 being the most predominant.

Fumonisins are mainly produced by the maize pathogens *Fusarium proliferatum* and

Fusarium moniliforme. Fumonisin consumption by horses can cause equine

leucoencephalomalacia (ELEM) (Kellerman *et al.*, 1990) and by pigs can cause porcine

pulmonary edema (PPE) (Osweiler *et al.*, 1992; Colvin and Harrison, 1992). Consumption of

FBs has been associated with other diseases such as embryopathogenicity in chicken (Javed

et al., 1993), developmental toxicity in hamsters (Floss *et al.*, 1994), and kidney toxicity and

liver cancer in rats (Gelderblom *et al.*, 1991; Voss *et al.*, 1993). The effects of FBs on

humans are not known. However, epidemiological studies have suggested a correlation

between high levels of FBs in corn consumed by humans and human esophageal cancer

(Syndenham *et al.*, 1990; Rheeder *et al.*, 1992; Chu and Li, 1994). Fumonisin B_1 is a

probable human class 2B carcinogen (IARC, 1993).

Fumonisin B_1 has been labeled using [^{14}C]-acetate or [^{14}C]-methionine in cultures of

F. moniliforme or *F. proliferatum* in liquid medium (LM) (Norred *et al.*, 1993; Blackwell *et*

al., 1994; Lebepe-Mazur, 1993). Norred et al. (1993) detected 80% and 2.3% of 1.4 μmole

[^{14}C]-FB$_1$/kg BW dosed by gavage in the feces and urine of fasted rats, respectively. The

livers, kidneys, and blood of these rats retained 0.6% of the [^{14}C]-FB$_1$-dose after 96 h.

Shephard et al. (1992) detected 100% and trace levels of 10.4 μmole [^{14}C]-FB$_1$/kg BW dosed

by gavage in the feces and urine of fed rats, respectively after 24 h. Trace levels of the [^{14}C]-

FB$_1$-dose were detected in the livers, kidneys, and blood of rats. A comparison of Norred's et

al. (1993) fasted rats to Shephard's et al. (1992) fed rats showed a greater urinary excretion

in fed rats compared to fasted rats suggesting enhanced absorption in fasted rats. Shephard et

al. (1994) reported 0.2% of a [^{14}C]-FB$_1$-dose in the bile of fed rats dosed by gavage with 10.4

μmole [^{14}C]-FB$_1$/kg BW after 24 h. Hopmans et al. (1997) administered 0.69, 6.93, or 69.3

μmoles of FB$_1$/kg BW to fed rats resulting in 7.4, 1.2, and 0.5% of the FB$_1$ dose was excreted

in the urine, respectively.

Previous attempts to detoxify FB$_1$ by ammoniation and dry heat treatments have been

unsuccessful (Norred et al., 1991; Dupuy et al., 1993). Alkaline hydrolysis of the

tricarboxylic (TCA) groups of FB$_1$ produced hydrolyzed FB$_1$ (HFB$_1$) which was found to be

as toxic as FB$_1$ to rats fed a nutritionally adequate diet (Hendrich et al., 1993). However,

HFB$_1$ may be less toxic than FB$_1$ because HFB$_1$ was absorbed 2-fold more than FB$_1$ by rats

(Hopmans et al., 1997). Detoxification of FB$_1$ may occur by formation of the Schiff's base,

FB$_1$-FRU (Murphy et al., 1995). Lu et al. (1997) reported FB$_1$-FRU did not cause rat hepatic

cancer promotion. Hopmans et al. (1997) reported that FB$_1$-FRU had a greater urinary

excretion than FB$_1$ in rats. The lack of toxicity of FB$_1$-FRU could not be explained by its

lower absorption than FB$_1$ in rats. Norred et al. (1993) recovered 35% of the [^{14}C]-dose in

the feces from fasted rats dosed by iv with 6.2 nmole [^{14}C]-FB$_1$/rat after 96 h. Shephard *et al.*

(1992) recovered 66% of the [^{14}C]-dose in the feces from fed rats dosed intraperitoneally with

10.4 μmole [^{14}C]-FB$_1$/kg BW after 24 h. In Shephard *et al.* (1994), 67% of a [^{14}C]-dose was

detected in the bile of fed rats dosed intraperitoneally with 10.4 μmole [^{14}C]-FB$_1$/kg BW after

24 h and 0.2% of a [^{14}C]-dose was detected in the bile of fed rats dosed by gavage with 0.4

μmole [^{14}C]-FB$_1$/kg BW. The biliary excretion of the HFB$_1$ and FB$_1$-FRU have not been

previously investigated. The objective of this study was to compare the urinary, fecal, and

biliary excretion of 0.69 μmoles of [^{14}C]-HFB$_1$ and [^{14}C]-FB$_1$-FRU/kg BW to [^{14}C]-FB$_1$/kg

BW dosed by gavage by rats.

Materials and Methods

Reagents were from Fisher Scientific (St. Louis MO) unless otherwise stated. The

FB$_1$ produced from *Fusarium* cultures is considered class 2B carcinogens (IARC, 1993) and

caution should be taken during their handling. We assume that HFB$_1$ is a potential

carcinogen and should be treated accordingly.

Liquid cultures (500 ml) of *F. proliferatum* strain M5991 were prepared as in Dantzer

et al. (1996a) and inoculated into Myro liquid medium (LM) which had MgSO$_4$ reduced to

0.5 g/L and contained 1.00% (v/v) corn hull extract (modified Myro LM). The inoculum

culture was incubated for 4 days (d) on a rotary shaker at 220 rpm and 25°C. Aliquots (0.5

ml) of the *F. proliferatum* M-5991 inoculum culture were transferred into each of 3 rubber

stoppered 125 ml Erlenmeyer flasks containing 50 ml of modified Myro LM. Compressed

air was cleaned by passing through an enclosed 2 L plastic bottle containing air, then bubbled

through a series of 4 enclosed 2 L plastic bottles containing; 1L of 2N KOH, 2N KOH,

distilled water (dH_2O), and 2N H_2SO_4, respectively, passed through a moisture trap, and

filtered with a 0.2 µm inline filter. The purified air was bubbled through the F. proliferatum

inoculated culture flasks at 0.5 ml/min while the flasks were incubated on a rotary shaker

(220 rpm) at 20-25°C for 30 d. One ml aliquots were removed from the culture flasks every 7

d and stored at -20°C until FB analysis.

Iowa State University (ISU) Environmental Health and Safety guidelines for ^{14}C use

were followed throughout. Glassware was cleaned by soaking in Nochromix laboratory glass

cleaner (GODAX Laboratories, Inc., Takoma Park, MD) for >15 min. An aliquot containing

0.5 ml of a 4 d old F. proliferatum M-5991 LM culture was transferred into a rubber

stoppered 125 ml Erlenmeyer flask. The filtered purified air was bubbled into the flask at 0.5

ml/min and allowed to exit through Tygon tubing (3.2 or 4.8 mm ID x 6.4 mm OD; formula

R-3603; Norton Performance Plastics, Akron, OH) to a CO_2 trap consisting of two rubber

stoppered 20 x 150 mm test tubes assembled in series each containing 20 ml of 2N KOH.

The flask was incubated at 220 rpm and 20-25°C on a rotary shaker for 10 d and a 100 µl

aliquot was removed once for pre-labeling FB_1 quantification. The ethanol (EtOH)

containing 56 mCi/mmole of [1,2-^{14}C]-acetate (American Radiolabeled Chemicals Inc. St.

Louis, MO, Lot # 960214) was evaporated and the residue was redissolved in milli-Q water

(MqH_2O). Four equal aliquots of 0.25 mCi of [1,2-^{14}C]-acetate were transferred into the F.

proliferatum flask after 12, 15, 18, and 21 d to equal a total of 1.00 mCi [1,2-^{14}C]-acetate.

Preliminary work indicated EtOH decreased production of FB_1 and cell density by F.

proliferatum M-5991. The culture was agitated on a shaker at 220 rpm throughout and

harvested 13 d after [^{14}C] addition. The KOH-^{14}CO$_2$ trap tubes were replaced every 24 h after

the initial addition of [^{14}C] and counted in ScintiVerse BD scintillation fluid for 4 min using a

Packard liquid scintillation analyzer Model 1900TR (Packard Instrument Co., Downers

Grove, IL).

[^{14}C]-Fumonisin B$_1$ purification was performed according to the procedure of Dantzer

et al. (1996b) with a few modifications. Briefly, the 50 ml of [^{14}C]-FB$_1$ containing LM was

filtered through a Whatman #1 filter (Whatman Laboratory Division, Springfield Mill,

Maidstone, Kent) followed by 0.8 and 0.45 μm MSi filters (4.5 cm dia. nylon; Micron

Separations Inc., Westboro, MA). The [^{14}C]-FB$_1$ was fractionated on XAD-16, C$_8$, DEAE-

Sepharose, and C$_{18}$ columns. The XAD-16 column was a 30 x 2 cm glass column with 50 g of

Amberlite XAD-16 (Sigma Chemical Co., St. Louis MO). The column was washed with 150

ml of MqH$_2$O and the FB$_1$ was eluted with 150 ml of 50% acetonitrile (ACN). All other

purification procedures were the same as in Dantzer *et al.* (1996b). After elution of the [^{14}C]-FB$_1$

from the C$_{18}$ column, the sample was concentrated by rotary evaporation at 45°C, freeze-dried,

weighed, rehydrated in 50% ACN, and quantified for FB$_1$. Three aliquots, each containing 1.39

μmole [^{14}C]-FB$_1$, were rotary evaporated to dryness at 45°C and used to prepare [^{14}C]-FB$_1$,

[^{14}C]-HFB$_1$, or [^{14}C]-FB$_1$-FRU dosing treatments. For preparation of the [^{14}C]-FB$_1$ treatment,

an aliquot containing 1.39 μmole [^{14}C]-FB$_1$ was rehydrated in 10 ml of MqH$_2$O and stored at

-20°C until used for animal dosing. For preparation of the [^{14}C]-HFB$_1$ treatment, an aliquot

containing 1.39 μmole [^{14}C]-FB$_1$ was rehydrated in 5 ml MqH$_2$O and hydrolyzed in a marble

capped test tube (12.5 x 1.5 cm) with 1 ml of 2N KOH for 2 h in boiling H$_2$O. The pH of the

HFB$_1$ solution was adjusted to <3 using 12N HCl and loaded onto a PrepSep P479-C$_{18}$

extraction column (Fisher Scientific). The column was washed with 20 ml of MqH$_2$O and

the HFB$_1$ was eluted with 10 ml MeOH (Hopmans *et al.*, 1997). The MeOH was removed

from the [^{14}C]-HFB$_1$ by rotary evaporation at 45°C. The [^{14}C]-HFB$_1$ was rehydrated in 10 ml

of MqH$_2$O and stored at -20°C until used for animal dosing. For preparation of the [^{14}C]-FB$_1$-

FRU treatment, an aliquot containing 1.39 μmole [^{14}C]-FB$_1$ was rehydrated in 10 ml of 0.05 M

K$_2$HPO$_4$ at pH 7 containing 0.1 M D-fructose and heated at 80°C for 48 h (Hopmans *et al.*,

1997). The volume of the heated FB$_1$-FRU solution was adjusted to 10 ml with MqH$_2$O and

stored at -20°C until used for animal dosing.

The concentrations of [^{14}C]-FB$_1$ and -HFB$_1$ were estimated by HPLC o-

pthaldialdehyde (OPA) fluorometry using a Turner fluorometer (Corning 7-60 primary filter

and Wratten 2a secondary filter, 15 μl flow cell) (Dantzer *et al.*, 1996b). Purity of [^{14}C]-FB$_1$

was determined by comparing the HPLC-OPA quantified amounts to the freeze-dried

weights of the purified [^{14}C]-FB$_1$ sample. A purified sample of FB$_1$ was used to generate a

FB$_1$ standard curve (Dantzer *et al.*, 1996b). The [^{14}C]-HFB$_1$ sample was quantified for HFB$_1$

by comparing the HPLC-OPA quantified amounts to a HFB$_1$ standard curve. The HFB$_1$

standard was generated in our lab according to the procedure of Hopmans *et al.* (1997). The

[^{14}C]-FB$_1$-FRU sample was analyzed for residual free [^{14}C]-FB$_1$ by HPLC-OPA.

The animal protocol and use was approved by the ISU Animal Care Committee.

Eighteen Fisher 344/NHsd rats, 9 male and 9 female at 7-8 and 9-10 wk of age, respectively,

with a BW range of 135-160 g were used for the urine and fecal excretion study. All rats

were housed individually and fed AIN 93M diet (Reeves *et al.*, 1993) and tap water *ad lib* for

1 wk with a 12 h light cycle. Following the 1 wk acclimation period and at the end of a light

cycle. 3 male and 3 female rats were administered 0.69 μmole [^{14}C]-FB$_1$, [^{14}C]-HFB$_1$, or

[^{14}C]-FB$_1$-FRU/kg BW by gavage. The rats were housed individually in metabolic cages and

fecal and urine samples were collected at 12 h intervals. At 84 h, the rats were sacrificed by

CO_2 asphyxiation, blood was drawn by heart puncture, and hearts, livers, lungs, kidneys, and

brains were removed for [^{14}C] analysis.

Nine female 15 wk old Sprague-Dawley rats with a BW range of 240-270 g were used

for the biliary excretion study. All rats were housed individually and fed AIN 93M diet

(Reeves *et al.*, 1993) and tap water *ad lib* for 1 wk with a 12 h light cycle. Following the 1

wk acclimation period and at the end of a light cycle, the rats were anesthetized with 5 ml of

urethane (Sigma) solution (0.25 g urethane/ml sterile H_2O)/kg BW intraperitoneally and their

bile ducts were surgically cannulated with a 24 cm long piece of silicone rubber tubing (0.3

mm ID x 0.6 mm OD; BrainTree Scientific Inc., Braintree, MA). Following cannulation, the

tubing was fed through a large bore needle placed in the right side of the abdomen of each

rat. After passing the tubing through the needle, the needle was withdrawn and the abdomen

surgically closed. Immediately after cannulation, 3 rats were gavaged with 0.69 μmole [^{14}C]-

FB$_1$, [^{14}C]-HFB$_1$, or [^{14}C]-FB$_1$-FRU/kg BW and placed on their left sides on a hot water

heating pad set at medium power. Bile was collected into 1.5 ml graduated micro centrifuge

tubes every 30 min until the rats expired or at sacrifice 9.5 h after dosing. Livers, kidneys,

stomach wash, stomach tissue, and the 1st, 2nd, and 3rd sections (I, II, and III) of the intestines

were collected after the rats expired or at sacrifice 9.5 h after dosing. All samples were

stored at -20°C until analyzed.

Fecal samples, oven-dried at 60°C overnight, and intestinal tissues, frozen with liquid

N_2, were ground in a porcelain mortar and pestle. Ground fecal and intestinal tissues,

stomach tissues, stomach washes, kidneys, livers, lungs, blood, and brains were separately

blended with a tissue homogenizer (Model TR-10, Tekmar Co., Cincinnati, OH) at 60%

power for 0.5 to 1 min in 5 ml MqH_2O, brought to a known volume (10 to 35 ml) with

MqH_2O, and quantified for [^{14}C] on a scintillation analyzer. Quenching of [^{14}C] was

evaluated by measuring [^{14}C] in 1 ml fecal, urine, liver, kidney, or dH_2O solutions or 0.5 ml

of intestinal tissue, stomach wash, stomach tissues, or dH_2O, with and without 0.1 ml ACN

containing 18,400 dpms from 0.02 μmoles of [^{14}C]-FB_1. The quenching % was used to

correct data from each sample.

The statistical treatment of the urine and fecal excretion of the FB forms, [^{14}C]-FB_1,

[^{14}C]-HFB_1, and [^{14}C]-FB_1-FRU, were a completely randomized design of 18 rats. Three

male and 3 female rats were randomly assigned to each treatment. The statistical protocol of

the biliary excretion of the FB forms, [^{14}C]-FB_1, [^{14}C]-HFB_1, and [^{14}C]-FB_1-FRU, were a

completely randomized design of 9 rats. Three female rats were randomly assigned to each

treatment. Differences among treatment means were assessed by a students t-statistic with p

≤ 0.05 as level of significance using the SAS package (V 6.03, 1995, Cary, NC).

Results and Discussion

Log-phase production of FB_1 by *F. proliferatum* culture occurred during 10-14 d

followed by a linear increase in FB_1 through 31 d yielding 1200 μmoles FB_1/L (Fig. 1).

There was an average production of 0.18 μCi $^{14}CO_2$/h within the first 24 h period after each

addition of 250 μCi [^{14}C]-acetate (Fig. 1). The production of $^{14}CO_2$ decreased to undetectable

levels 3 d after addition of the [^{14}C]-acetate. The culture was harvested at 24 d. The FB_1 was

purified to >95% purity with a yield of 24 μmoles and a specific activity of 1.7 mCi/mmole.

[^{14}C]-Fumonisin B_1 measurement in biological material allows for improved ability to

monitor FB_1 compared to HPLC-fluorescence methods. However, quantifying for [^{14}C]-FB_1

will not reveal any information on metabolic modification of FB or its related forms.

The doses for the urine and fecal excretion study contained 0.14 μmole of [^{14}C]-FB_1,

[^{14}C]-HFB_1, or [^{14}C]-FB_1-FRU/ml MqH_2O with specific activities of 1.7, 1.2, and 1.8

mCi/mmole, respectively. The lower specific activity of [^{14}C]-HFB_1 suggested that some of

the TCA groups had been labeled during [^{14}C]-FB_1 production and were removed by

hydrolysis to produce [^{14}C]-HFB_1.

Dosing of rats during the urine and fecal excretion study occurred at the end of a light

cycle suggesting the animals were not actively feeding for 12 h prior to dosing. The

quenching analysis indicated that blood had the highest average quenching at 86% followed

by liver at 28% of the added [^{14}C]-FB_1. All other constituents had little quenching (Table 1).

The data on time course of excretion of [^{14}C] in urine of male and female rats, for each FB

form, were combined because they were not significantly different except for urinary

excretion of HFB_1 during 0-12 h after dosing (Fig. 2). There was 15.0, 4.4, and 0.6% of the

[^{14}C]-dose excreted in the urine dosed with 0.69 μmole [^{14}C]-HFB_1, [^{14}C]-FB_1-FRU, and

[^{14}C]-FB_1/kg BW, respectively (Table 2). The average half-life of the FB compounds

retained by these rats was 10 h. The urinary excretion of [^{14}C]-HFB_1 persisted up to 60 h

after dosing with a maximum of [^{14}C]-HFB_1 being excreted between 12 and 24 h. The

excretion of [^{14}C]-HFB$_1$ resembled a one-compartment model of elimination. The elimination of [^{14}C]-FB$_1$-FRU persisted in the urine of these rats up to 24 h after dosing (Fig. 2). The urinary excretion of [^{14}C]-FB$_1$-FRU persisted up to 24 h with a maximum of [^{14}C]-FB$_1$-FRU being excreted between 0 and 12 h. The excretion of [^{14}C]-FB$_1$-FRU in these rats resembled a one-compartment model of elimination. There were only trace amounts of [^{14}C]-FB$_1$ excreted in the urine of these rats and most of that was excreted in the first 12 h after dosing (Fig. 3). Over 25-fold more [^{14}C]-HFB$_1$ was excreted in the urine of male and female rats than [^{14}C]-FB$_1$ suggesting that HFB$_1$ had a greater bioavailability. The greater bioavailability of HFB$_1$ than FB$_1$ with comparable toxicity (Hendrich et al., 1993) suggested that FB$_1$ was more toxic than HFB$_1$ at the cellular level to rats. Total excretion of [^{14}C]-FB$_1$-FRU in the urine of male and female rats was 8-fold greater than FB$_1$ suggesting that [^{14}C]-FB$_1$-FRU had a greater bioavailability than FB$_1$ in these rats (Table 2). Fumonisin B$_1$-FRU was a detoxified form of FB$_1$ in rats (Lu et al., 1997). However, its detoxification mechanism was not due to reduced absorption since its bioavailability was greater than that of FB$_1$ in rats. Our total urinary excretion of 0.69 μmoles HFB$_1$ and FB$_1$-FRU/kg BW compared to 0.69 μmoles FB$_1$/kg BW by male and female Fisher rats after 84 h were similar to the urinary excretion by male Fisher rats observed by Hopmans et al. (1997) after 96 h. Both studies reported the same total percent urinary excretion of HFB$_1$ and relative absorptions of HFB$_1$ and FB$_1$-FRU compared to FB$_1$. However, in the study by Hopmans et al. (1997) rats dosed with 0.69 μmoles of FB$_1$-FRU or FB$_1$/kg BW excreted 2-fold more percent total dose of FB$_1$-FRU and 14-fold more percent total dose of FB$_1$ in the urine than our rats. Hopmans et al. (1997) correlated FB$_1$ and FB$_1$-FRU to the amount of OPA-HFB$_1$ in

the hydrolyzed rat urine. Hydrolysis of urine was performed to free up the amine group on the FB_1 molecule for OPA binding. In our study, FB_1 and FB_1-FRU were correlated to the amount of [^{14}C] detected in the rat urine. The differences in these two studies may reflect the differences in analysis. Norred et al. (1993) found 2-3% of a gavaged dose of 1.4 μmole [^{14}C]-FB_1/kg BW was excreted in the urine of fasted Sprague-Dawley rats after 96 h suggesting that their fasted rats had a greater absorption of FB_1 than our fed rats. This apparent higher absorption by Norred's rats compared to our rats may have been attributed to differences in strain of rat or, more likely, in absorption of FBs between fasted and fed rats. In fed rats, solids may tie up the FBs and make them less available for GI tract absorption.

The data on the time course of fecal excretion of [^{14}C] in male and female rats, dosed with the same compound, were combined because they were not significantly different according to gender (Fig. 3). The pattern of fecal excretion of [^{14}C]-FB_1, [^{14}C]-HFB_1, and [^{14}C]-FB_1-FRU followed a normal excretion of a compound through the fecal route (Casarett and Doull, 1991) with maximum excretion of [^{14}C] from the 3 compounds to be recovered in the feces between 12 and 24 h. After 60 h, only trace amounts of [^{14}C] were recovered in the feces from these rats. Total fecal excretion was not significantly different between compounds or gender and averaged 90% recovery of total dose after 84 h (Table 2).

The total [^{14}C] recovered from the hearts, brains, livers. blood, kidneys, or lungs of rats dosed with 0.69 μmole [^{14}C]-FB_1, [^{14}C]-HFB_1, or [^{14}C]-FB_1-FRU/kg BW was not significantly different from 0 for all rats indicating that accumulation of these compounds did not occur after 84 h (Fig. 4, Table 2).

In the biliary excretion study, Sprague-Dawley rats were used to obtain rats big

enough for the canulation procedure. The same treatments were used for the biliary excretion

study as in the urinary excretion study. However, to obtain proper volumes, the 0.14 μmole

[^{14}C]-HFB$_1$ and [^{14}C]-FB$_1$-FRU/ml MqH$_2$O treatments were diluted with unlabeled 0.14

μmole HFB$_1$ or FB$_1$-FRU/ml MqH$_2$O which resulted in specific activities of 0.7 and 1.0

mCi/mmole, respectively. In the bile excretion experiment, all 9 of the cannulated female

rats survived for 4 h after dosing. Biliary excretion of 0.69 μmole [^{14}C]-FB$_1$, [^{14}C]-HFB$_1$, or

[^{14}C]-FB$_1$-FRU/kg BW by female rats were not significantly different with 1.35% of the total

[^{14}C]-dose excreted 4 h after dosing (Table 3). Biliary excretion of the 3 FB compounds

increased from 0 to 0.5% of the total dose per 0.5 h interval within 2 h after dosing (Fig. 5).

The 3 forms of FB continued to be excreted in the bile by the rats up to 9.5 h after dosing

(Fig. 6). The large error bars seen in the biliary excretion data may suggest the presence of

fluorescent compounds that have been previously observed in rat bile (Hicks *et al.*, 1984).

Only 2 rats from the [^{14}C]-FB$_1$ dose and 1 rat from the [^{14}C]-HFB$_1$ and [^{14}C]-FB$_1$-FRU doses

survived for 9.5 h. Shephard *et al.* (1994) recovered almost 7-fold less at 0.2% of the total

[^{14}C]-dose in the bile of cannulated rats gavaged with [^{14}C]-FB$_1$ compared to our rats.

However, Shephard's *et al.* (1994) rats were not under anesthesia during gavaging of [^{14}C]-

FB$_1$ or bile collection. A large portion of the [^{14}C]-dose of the 3 forms of FB$_1$ were recovered

in the stomach contents of our rats (41% of the total dose) indicating that only about 1/2 of

the dosed-FB compounds left the stomachs of these rats (Table 4). This could possibly be

because these rats were under anesthesia throughout the duration of the experiment which

may have resulted in a slower rate of GI tract absorption compared to Shephard's *et al.*

(1994) rats. The pooled stomach tissues, intestines, and bile samples from the rats contained

10 ± 8, 8 ± 9 and $3 \pm 4\%$ of the total dose, respectively. Kidneys and livers contained less

than 1% of the $[^{14}C]$-dose. Total recovery of the $[^{14}C]$-doses from these rats of the bile

excretion study averaged $63 \pm 17\%$.

Conclusions

The 25-fold greater absorption of $[^{14}C]$-HFB_1 than that of $[^{14}C]$-FB_1 in male and

female Fisher rats suggested that, once in circulation, HFB_1 was less toxic than FB_1, on a

molar basis, because both have been shown to be equally toxic on a dietary basis to rats

(Hendrich *et al.*, 1993). Detoxification of FB_1 by formation of FB_1-FRU was not the result of

decreased absorption since $[^{14}C]$-FB_1-FRU was absorbed 8-fold more than $[^{14}C]$-FB_1 by these

rats. These data compliment and extend the findings of Hopmans *et al.* (1997) suggesting

that HFB_1 or FB_1-FRU were absorbed more than FB_1 by the GI tract of rats. In addition,

there were no differences in biliary excretion of $[^{14}C]$-FB_1, $[^{14}C]$-HFB_1, or $[^{14}C]$-FB_1-FRU by

these rats. This finding suggests that the observed decrease in urinary excretion of FB_1

compared with HFB_1 or FB_1-FRU was due to a decreased absorption of FB_1 relative to HFB_1

or FB_1-FRU by the GI tract of these rats.

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Table 1. Detectability of [^{14}C] in biological samples spiked with [^{14}C]-FB$_1$.

SAMPLE	AVE DPM	PERCENT OF DOSE DETECTABLE
Urine	17,902	97
Feces	22,662	123
Lung	17,231	94
Heart	17,521	95
Brain	18,188	99
Liver	13,358	72
Blood	2,536	14
Kidney	16,897	92
Bile	18,763	102
Intestine	17,708	96

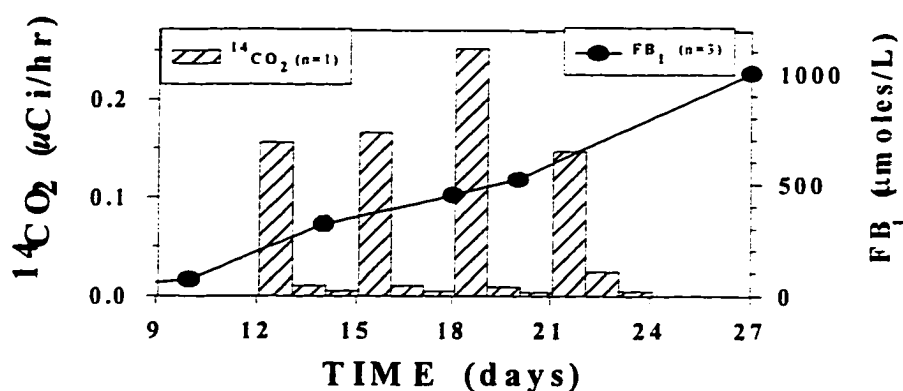Samples were spiked with 100 ml of acetonitrile containing 18,000 dpms from 0.02 mmoles of [^{14}C]-FB$_1$.

Fig. 1. Production of $^{14}CO_2$ (μCi/h) and FB_1 (μmoles/L) by *Fusarium proliferatum* M-5991. Bar plot represents production of $^{14}CO_2$ (μCi/h) by *F. proliferatum* M-5991 in 50 ml of modified Myro liquid medium (LM) spiked with 0.25 mCi [^{14}C]-acetate after 12, 15, 18, and 21 d of incubation. Line plot represents FB_1 (μmoles/L) produced by *F. proliferatum* M-5991 in 50 ml of modified Myro LM. Error bars represent ± 1 standard deviation of 3 replicates.
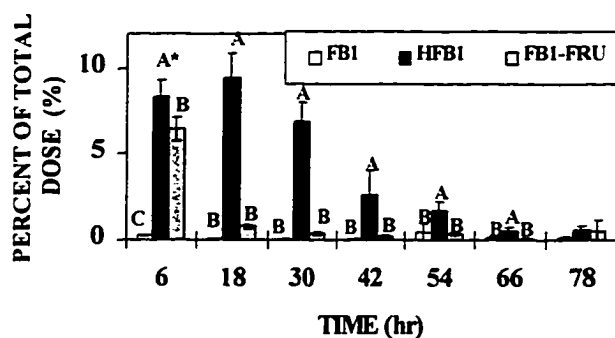

Fig. 2. Percent excretion in urine of total [^{14}C]-dose per 12-h interval by groups of 3 male and 3 female rats dosed with 0.69 μmole [^{14}C]-FB_1 or [^{14}C]-HFB_1 or [^{14}C]-FB_1-FRU/kg BW. Error bars represent ± 1 standard deviation (n=6). *There was a significant difference between male and female rats for treatment-time interval. Data within a time interval with different superscripts are statistically different ($p \leq 0.05$).

Table 2. Percent recovery of total ^{14}C of 0.69 μmoles of [^{14}C]-FB$_1$ or [^{14}C]-HFB$_1$ or [^{14}C]-FB$_1$-FRU/kg BW in male and female rats after 84 h.

	PERCENT TOTAL DOSE							
	—URINE—		—FECES—		—TISSUES—		TOTAL --RECOVERY--	
DOSE	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
[^{14}C]-FB$_1$	0.44^A	0.72^A	84.98	95.49	0.28	2.72	85.7	98.9
[^{14}C]-HFB$_1$	12.83^C	17.30^D	86.81	91.42	0.14	1.31	99.8	110.0
[^{14}C]-FB$_1$-FRU	4.18^B	4.59^B	86.20	97.00	0.19	0.42	90.6	102.0

Different superscripts are significantly different (p≤0.05). MSE$_{urine}$ = 2; MSE$_{feces}$ = 89; MSE$_{tissues}$ = 2; MSE$_{recovery}$ = 81. Data within feces, tissues, or total recovery are not significantly different (p≤0.05).
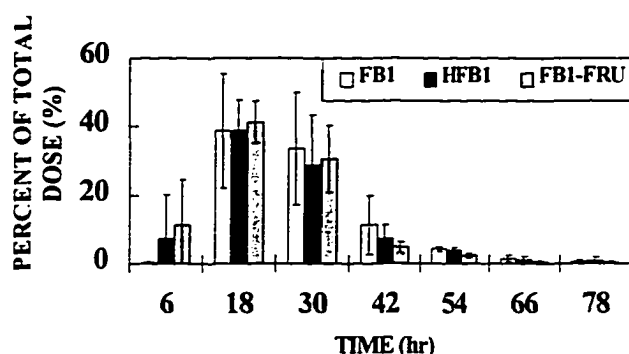

Fig. 3. Percent excretion in feces of total [^{14}C]-dose per 12-h interval by groups of 3 male and 3 female rats dosed with 0.69 μmole [^{14}C]-FB$_1$ or [^{14}C]-HFB$_1$ or [^{14}C]-FB$_1$-FRU/kg BW. Error bars represent ± 1 standard deviation (n=6). Data within each time interval were not significantly different (p≤0.05).

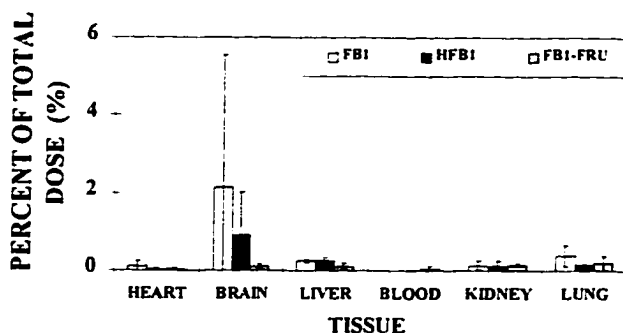Fig. 4. Percent tissue accumulation of total ^{14}C dose by groups of 3 male and 3 female rats (n=6) dosed with 0.69 μmole [^{14}C]-FB$_1$ or [^{14}C]-HFB$_1$ or [^{14}C]-FB$_1$-FRU/kg BW. Error bars represent ± 1 standard deviation. Data within each tissue group were not significantly different (p≤0.05).
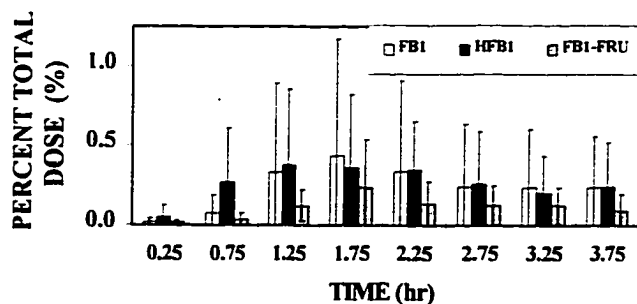

Fig. 5. Percent biliary excretion of total ^{14}C dose per 0.5 hour interval by groups of 3 female rats dosed with 0.69 μmole [^{14}C]-FB$_1$ or [^{14}C]-HFB$_1$ or [^{14}C]-FB$_1$-FRU/kg BW. Error bars represent ± 1 standard deviation (n=3).
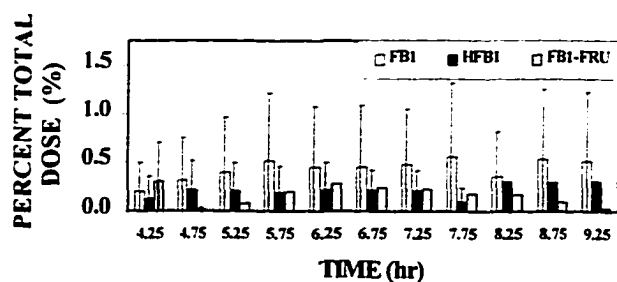
Fig. 6. Percent biliary excretion of total ^{14}C dose per 0.5 hour interval by female rats dosed with 0.69 μmole [^{14}C]-FB$_1$ or [^{14}C]-HFB$_1$ or [^{14}C]-FB$_1$-FRU/kg BW. Bars with error bars represent means ± range (n=2). Bars without error bars represent n=1.

Table 3. Biliary excretion of [^{14}C]-dose by female rats gavaged with 0.69 μmole [^{14}C]-FB$_1$ or [^{14}C]-HFB$_1$ or [^{14}C]-FB$_1$-FRU/kg BW 4 hours after dosing.

	PERCENT BILIARY EXCRETION OF [^{14}C]-DOSE AFTER 4 HOURS	
DOSE	AVERAGE	STANDARD DEVIATION
FB$_1$	1.55	2.51
HFB$_1$	1.71	1.99
FB$_1$-FRU	0.80	0.82
n=3		

Table 4. Percent of total [^{14}C] dose (%) recovered in constituents from female Sprague-Dawley rats dosed with 0.69 μmoles of [^{14}C]-FB$_1$, -HFB$_1$, or -FB$_1$-FRU and the duration (hr) until expiration of each rat after dosing.

	PERCENT OF TOTAL [^{14}C]-DOSE (%)								
	FB$_1$			HFB$_1$			FB$_1$-FRU		
	RAT			RAT			RAT		
PARAMETER	1	2	3	1	2	3	1	2	3
BILE	11.1	0.1	0.2	6.6	0.2	1.1	2.9	0.4	0.3
KIDNEYS	0.9	0.0	0.1	0.1	0.0	0.8	0.4	0.2	0.0
LIVER	1.4	0.0	0.9	0.2	0.0	0.5	0.0	0.0	0.0
STOMACH WASH	17.9	65.7	34.1	21.4	87.6	16.2	34.4	46.2	42.4
STOMACH TISSUE	16.8	3.7	7.6	7.0	7.3	4.1	29.5	4.4	7.0
INT I TISSUE	2.4	0.0	1.8	14.5	0.0	0.5	5.1	15.8	1.8
INT II TISSUE	0.0	0.1	0.0	2.5	0.0	4.9	0.0	0.0	0.0
INT III TISSUE	0.0	10.2	0.0	0.6	0.4	22.8	0.0	0.2	0.0
TOTAL	50.0	80.0	45.0	53.0	96.0	51.0	72.0	67.0	52.0
DURATION (hr)	9.0	9.0	4.0	9.0	7.0	4.0	9.0	4.5	3.5

CHAPTER 6. GENERAL CONCLUSIONS

A. Summary

The structure of fumonisin B_1 (FB_1) suggests that it is a symmetrical molecule that may be able to fold into different configurations and is water soluble. From a toxicologist's standpoint, the active groups on a FB molecule include the tricarboxylic acid groups, primary amine group, and a C20 backbone with 1 to 3 hydroxyl groups and 2 methyl groups attached. Fumonisin B_1 has been shown to be only slightly absorbed by the gastrointestinal (GI)-tract of animals. However, it is capable of causing major health problems such as leucoencephalomalacia, pulmonary edema, liver cancer, or kidney disease in different animals (Kellerman et al., 1991; Osweiler et al., 1992; Gelderblom et al., 1991; Voss et. al., 1993). In studies described in this dissertation, less than 2% of the $[^{14}C]$-FB_1-dose was absorbed by the GI tract of rats. Of the 2% absorbed dose, it is estimated that 25% was excreted in the urine and 75% was excreted in the bile. Altering the form of $[^{14}C]$-FB_1 to $[^{14}C]$-hydrolyzed FB_1 ($[^{14}C]$-HFB_1) or $[^{14}C]$-FB_1-fructose ($[^{14}C]$-FB_1-FRU) resulted in an increased absorption by rats but not in an increased biliary excretion. The $[^{14}C]$-HFB_1 form may be found in foods such as masa and tortilla flour and was absorbed 30-fold more in the GI tract of rats than was $[^{14}C]$-FB_1. Based on these data and their similar dietary toxicity to rats (Hendrich et al., 1993), dietary HFB_1 may be less toxic than dietary FB_1 on a cellular basis. Another form of $[^{14}C]$-FB_1 studied here was $[^{14}C]$-FB_1-FRU, which can be formed by heating $[^{14}C]$-FB_1 with fructose at pH 7. This form was found not to be a cancer promotor when fed to rats (Lu et al., 1977). The mode of detoxification of FB_1-FRU could have been a

decreased absorption by the GI tract of rats. However. Hopmans *et al.* (1997) reported FB_1-FRU was absorbed more by the GI tract of in rats than was FB_1. The decreased toxicity of FB_1-FRU compared with FB_1 was not based on absorption. In this study, $[^{14}C]$-FB_1-FRU was absorbed 10-fold more than $[^{14}C]$-FB_1 by rats, confirming the findings of Hopmans *et al.* (1997). If FB_1-FRU can be shown not to cause some of the other animal toxicities such as ELEM and PPE, then the process of forming FB_1-FRU from FB_1 could be used to treat animal feeds that contain FBs.

These $[^{14}C]$-FB_1, $[^{14}C]$-HFB_1, and $[^{14}C]$-FB_1-FRU animal experiments required production and purification of $[^{14}C]$-FB_1. This required optimization of production, purification, and radiolabeling of FB_1. The production of FB_1 was optimized by incubating a FB_1-producing fungi, *Fusarium proliferatum* M-5991, in a modified Myro liquid medium (LM). The modification of the Myro LM included the addition of 1% (v/v) corn hull extract (CHE) which enhanced the overall growth characteristics of the fungi resulting in a 3-fold increase in the production of FB_1. The production of FB_1 in a large scale 12 L fermentation vessel was carried out in a serial batch fermentation in which the cell mass was retained from batch to batch. The serial batch fermentation demonstrated that the production of FBs could be scaled up and optimized to increase the production of FB_1 by *F. proliferatum*. This procedure also produced FB_1 in less time than shaker flasks because the vessel did not require cleaning after each fermentation batch. Possibly, the serial batch fermentation could have been improved. In this experiment, the CHE was only added to the first batch because purification of FB_1 produced from LM enhanced with CHE was not investigated. However, no purification problems were observed when the CHE was used in the production medium.

The basic chromatography method existed prior to this study but required major optimization and the addition of an anion exchange column. Radiolabeling of FB_1 with [^{14}C] included the addition of 1 mCi [^{14}C]-acetate to a 12 d old culture of *F. proliferatum* M-5991 in 50 ml of LM spread out over 10 d and resulted in the production of radiolabeled FB_1 with a specific activity of 1.7 mCi/mmole. This specific activity could be increased by adding all of the [^{14}C]-acetate at one time which would decrease the amount of non labeled FB_1 obtained after harvesting. However, due to the low recovery of FB_1 from the purification procedure, a greater loss of the [^{14}C]-FB_1 may have occurred.

B. Recommendations for Future Research

This study answered some questions concerning the absorption of 3 forms of FB_1 in rats. However, the absorption of similar forms of FB_2, FB_3, and FB_4 need to be investigated. Some *Fusarium* strains have been shown to produce FB_2, FB_3, and FB_4 preferentially over that of FB_1. What are the effects that these other FBs and their various forms have on animals? Can they be detoxified? Can the detoxified FB_1-FRU, which is absorbed more than FB_1, cause ELEM in horses?

There is strong evidence suggesting that FBs are toxic, exist in human and animal foods, and pose a risk to the health of humans and animals if consumed. However, the levels of FBs and the forms of FBs found in foods have not been regulated. Determining the safe levels of these compounds in foods needs to be completed. This would also require enhanced improvements in the detection of FBs from foods. The ELISA methods offer the ability to screen large numbers of samples at one time. However, ELISA methods are not as accurate

in quantifying levels of FBs in food systems as are the conventional HPLC-fluorometric

methods. In summary, other forms of these mycotoxins should be investigated for their

toxicity, acceptable levels in foods need to be determined, and quick. accurate methods for

detection of FBs need to be developed.

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BIOGRAPHICAL SKETCH

William R. Dantzer was born on June 28, 1960 in Columbus, Ohio. He received the Bachelor of Science in Food Science and Technology from The Ohio State University in 1991 and the Master of Science in Food Science and Technology from the University of Massachusetts (UMASS) in 1993. He has served as a Research Assistant at UMASS and at Iowa State University. At UMASS he worked on evaluating the effects of external bacteria on toxin production by dinoflagellated "Red Tide" algae contradicting previous reports that the toxin was produced by bacteria associated with the algae. While at ISU, he studied the absorption of 3 forms of fumonisin B_1 in rats as reported in this dissertation.