Sensitive Immunoassay for Fumonisin B_1 in Corn

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Polyclonal antibodies against fumonisin B_1 (FB_1) were produced in rabbits after immunization with FB_1 conjugates. Protection of the amino group prior to coupling the carboxylic acid of FB_1 to human serum albumin, and then deprotecting the amine before immunization, generated high-affinity antibodies. The antibodies produced were specific to fumonisins and their hydrolyzed products (HFB_1, HFB_2) but not to any other mycotoxins commonly found in grains. The relative cross-reactivities of FB_1, fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), HFB_1, and HFB_2 were 100%, 55%, 13%, 11%, and 3%, respectively. Using these antibodies, a sensitive indirect competitive ELISA was developed. The detection limit and IC_{50} for FB_1 in water were 0.05 and 0.66 ng/mL, respectively. The linear range was determined to be 0.05−0.75 ng/mL. The method was applied directly to 75% methanol extracts of corn. Recoveries of FB_1 were better than 87% from 10 to 10 000 ng/g at five levels of fortification. The detection limit in corn was 5 ng/g.

Keywords: *Fumonisins; mycotoxins; ELISA; immunoassay; corn*

INTRODUCTION

Progress is being made toward faster and cheaper analyses of human food and animal feed for mycotoxins such as fumonisins. Fumonisins (Figure 1) are toxic secondary metabolites produced in quantity by naturally occurring *Fusarium* fungi in corn, in particular *Fusarium moniliforme* and *Fusarium proliferatum* (Miller et al., 1995). The presence of these mycotoxins in foods and feeds is potentially hazardous to humans and animals. Fumonisins have been epidemiologically and experimentally linked to leukoencephalomalacia in horses (Gelderblom et al., 1988), pulmonary edema in swine (Kriek et al., 1981), and organ damage and failure to thrive in poultry (Brown et al., 1992). They are hepatocarcinogenic in rats (Gelderblom et al., 1988) and are also suspected of causing human esophageal cancer (Marasas et al., 1988). Inhibition of sphingosine *N*-acyltransferase and *de novo* sphingolipid biosynthesis is proposed as one mechanism of action for fumonisin B_1 (Merrill et al., 1993).

Although it is difficult to eliminate fumonisins from our food chain, it is possible to decrease the risk of exposure through vigorous programs of monitoring and the surveillance of these toxins in our food supplies. Fumonisins B_1, B_2, and B_3 (FB_1, FB_2, FB_3) are analyzed typically by chromatographic methods (for a recent review, see Scott, 1993), including thin layer chromatography (Shelby et al., 1994), liquid chromatography (LC) (Scott and Lawrence, 1992; Shephard et al., 1990), LC−mass spectrometry (LC−MS) (Chen et al., 1992), gas chromatography−MS (GC−MS) (Plattner et al., 1990), and GC−Fourier transform infrared spectroscopy (GC−FTIR) (Young and Games, 1994). More recently,

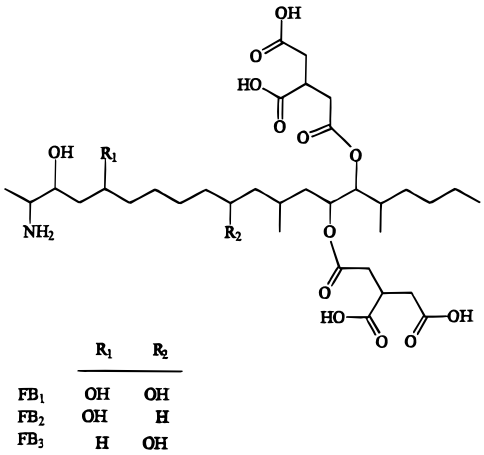

Figure 1. Chemical structures of the fumonisins FB_1, FB_2, and FB_3.

a capillary zone electrophoresis technique for FB_1 has also been described (Maragos, 1995).

Analyses of fumonisins by chromatographic methods are difficult tasks especially when only trace amounts of the toxins are present in the samples. However, rapid progress in the development of immunoassays for mycotoxins, including FB_1, has been evident in recent years, and these assays have gradually gained acceptance as viable alternative analytical methods (for reviews, see Chu, 1992; Pestka, 1988). Several fumonisin specific antibodies, both polyclonal and monoclonal, have been produced (Elissalde et al., 1995; Usleber et al., 1994; Fukuda et al., 1994; Azcona-Olivera et al., 1992a,b). Chu and his associates (Chu et al., 1995) reported the production of anti-idiotype and anti-anti-idiotype antibodies against FB_1. A rapid dipstick and immunofiltration assays for FB_1 in corn-based food are also known (Schneider et al., 1995). For antigens, these researchers all used glutaraldehyde or sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate to link the FB_1 amino functionality to the carrier proteins. We report, herein, an alternative approach of linking FB_1 to a carrier protein through one of its carboxylic acid groups.

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The amino group was protected prior to the carbodiimide coupling reaction and deprotected thereafter to preserve the suggested active form of FB_1. Gelderblom et al. (1993) demonstrated that a free amino group in a fumonisin molecule is essential for its biological activities. We anticipated that the amino moiety in FB_1 might be an essential epitope for high-affinity antibodies. The application of these polyclonal antibodies to enzyme-linked immunosorbent assay (ELISA) is described.

EXPERIMENTAL PROCEDURES

Materials. Safety note: Fumonisins are suspected carcinogens and should be handled with care. FB_1, FB_2, FB_3, and $HFB_1(AP_1)$ (>98% purity) were produced as described by Blackwell et al. (1994). HFB_1 was also obtained from W. T. Shier (University of Minnesota), and HFB_2 was a gift from W. C. A. Gelderblom (South African Medical Research Council). Deoxynivalenol, zearalenone, T-2 toxin, aflatoxin B_1, ochratoxin A, sphingosine, and sphinganine were purchased from Sigma Chemical Co. (St. Louis, MO).

In this study, cholera toxin (CT), bovine serum albumin (RIA grade), ovalbumin, human serum albumin (HSA), goat anti-rabbit IgG horseradish peroxidase conjugate (second antibody), Tween 20, *o*-phenylenediamine dihydrochloride (OPD), and glycerol were purchased from Sigma Chemical Co., St. Louis, MO. Glutaraldehyde, 1-ethyl-3-[(dimethylamino)propyl]carbodiimide hydrochloride (EDC), tricarballylic acid, and BOC-ON [2-[[(*tert*-butoxycarbonyl)oxy]imino]-2-phenylacetonitrile] were obtained from Aldrich Chemical Co. (Milwaukee, WI). Freund's complete and incomplete adjuvants were obtained from Gibco, Grand Island, NY. Dialysis tubing (10 mm in diameter with a 12000−14000 molecular weight cutoff) was purchased from Spectrum Medical Industries Inc., Los Angeles, CA. Flat-bottomed polystyrene microtiter plates were obtained from Dynatech Laboratories, Inc., Chantilly, VA. All solvents were of HPLC grade.

Buffers. Phosphate-buffered saline (PBS), pH 7.4, contained 20 mmol of NaH_2PO_4 and 140 mmol of NaCl oer liter of deionized water. Washing buffer (PBS-T) consisted of 0.1% Tween 20 in PBS. Coating buffer (pH 9.6) contained 13 mmol of Na_2CO_3 and 35 mmol of $NaHCO_3$, while citric buffer (pH 5.0) consisted of 51 mmol of Na_2HPO_4 and 24 of mmol citric acid per liter of deionized water. The substrate consisted of 17.5 mg of OPD and 10 μL of 30% H_2O_2 in 25 mL of citric buffer.

Instrumentation. Microtiter plates were washed with PBS-T using a Bio-Rad Microplate washer with five wash and soak cycles programmed for 8 s duration each. A 12-channel pipetter was used for dispensing liquids. Optical densities (OD) of microtiter wells were measured on a dual-beam Titertek Multiscan MCC instrument with a 492 nm sample filter and a 620 nm reference filter. Data were transmitted to a spreadsheet program for analysis. The instrument was checked periodically by a Spectrocheck plate and software (QC Technology, NY).

Immunogen. Three antigens of FB_1 were made. Two were conjugated via the amino group, while the other was through the carboxylic acid functionality.

Amino Conjugation: FB_1-a-CT. FB_1 was conjugated to glutaraldehyde-modified cholera toxin for use as immunogen and to ovalbumin for use as coating protein according to Azcona-Olivera et al. (1992a).

FB_1-a-HSA. The other immunogen was prepared by conjugating FB_1 to human serum albumin instead of cholera toxin using the same glutaraldehyde procedure, except the resultant reaction mixture was dialyzed in running water for 48 h before storing it at −20 °C.

Carboxylic Conjugation: FB_1-c-HSA. For the production of an alternative FB_1 antigen, the primary amino group of FB_1 was protected by BOC-ON prior to the conjugation of the carboxylic acid to the carrier protein. The protecting group was subsequently removed by acidolysis (Itoh et al., 1977; Meienhofer, 1985). Briefly, triethylamine (22 μmol, 1.62 mL) was added dropwise to a solution of FB_1 (20 μmol, 14.4 mg) and BOC-ON (20 μmol, 4.9 mg) in 50% aqueous 1,4-dioxane (4 mL) while stirring in an ice bath. The reaction was continued at room temperature. After 5 h, the reaction mixture was extracted three times with ethyl ether (15 mL × 3) to remove byproducts. The aqueous layer was adjusted to pH 7, and the volume was reduced to about 1 mL in a rotary evaporator at 30 °C. The resultant mixture was chilled to 4 °C; 1 mL of 50 μM cold sodium dihydrogen phosphate, pH 7.0, and 50 mg of EDC were added to the mixture. After mixing, human serum albumin (400 mmol, 28 mg) in 3 mL of the same phosphate buffer was added to the N-protected FB_1 mixture. The reaction was allowed to proceed for another 18 h at 4 °C on an orbital mixer. The resultant conjugate was dialyzed first in acidic water, adjusted with acetic acid to pH 4, for 24 h at 4 °C to remove the BOC-ON protecting group and then against running water for an additional 24 h. The FB_1 conjugate was stored frozen at −20 °C in 1 mL aliquots.

Immunization. Four groups of four male New Zealand white rabbits, 2.5 kg, were used to raise antibodies against FB_1. All rabbits received 0.5 mL of 1 mg/mL immunogen subcutaneously at four sites. Two groups were immunized and boosted with FB_1-a-CT or FB_1-a-HSA conjugate mixed with 50 μg of CT as adjuvant. For the other two groups, FB_1-a-HSA and FB_1-c-HSA immunogens were emulsified in an equal volume of Freund's complete adjuvant. Booster injections were given at 4 week intervals, substituting incomplete adjuvant for complete adjuvant.

Serum titers were monitored. Animals producing the highest titers and most sensitive inhibition curves were exsanguinated under anesthesia. Optimal immunization occurred from 5 to 7 months after the initial priming injection. Sera were kept frozen at −20 °C in 200 μL aliquots. Once thawed, an equal volume of 50% glycerol in water was added, and the solution was stored at −20 °C at all times. In this study, the glycerolated antibodies can be used for at least 6 months with no loss of activity.

Coating Protein. FB_1-ovalbumin coating proteins were prepared by a glutaraldehyde and sodium borohydride coupling reaction according to Azcona-Olivera et al. (1992a). Portions of 200 μL of coating protein at 1 μg/mL plus 10 μg/mL ovalbumin were used to sensitize the 96-well plates, in a manner previously reported (Newsome et al., 1993). These sensitized plates, which can be stored up to 6 months at −20 °C in this study, were used for all the FB_1 analyses.

Immunoassay. The ELISA procedure was similar to the one previously reported (Newsome et al., 1993). Briefly, a 1 mL aliquot of antiserum diluted 1:30000 with 0.1% BSA in PBS diluent was added to 25 μL of sample or standard. After mixing and incubation at 4 °C for 60 min, 200 μL was added to the wells of the sensitized plate in triplicate. After a further 30 min incubation at 4 °C and washing, a second-antibody horseradish peroxidase conjugate was added. Following an additional incubation step at room temperature for 30 min and washing, the substrate OPD and H_2O_2 were added. Thirty minutes later, the color reaction was stopped by the addition of H_2SO_4 and the optical densities were read at 492 nm. The FB_1 was determined by a least-squares plot of the logit of the optical density (OD) against the log of the concentration of the standards. The standard curve consisted of eight concentrations of FB_1 (0 and 0.05−0.75 ng/mL) in H_2O. Stock solutions of FB_1 standard (1 and 0.1 mg/mL) were dissolved in acetonitrile:water (3:1) and stored in a refrigerator.

Sample Preparation. A 10 g subsample of previously ground corn was homogenized in 100 mL of aqueous methanol (75% MeOH) using a Polytron for 1 min at medium speed and then agitated (orbital shaker, 100 rpm/min) for 30 min. Samples were centrifuged at 1600*g* for 30 min. The supernatant was filtered through Whatman No. 1 paper, and about 3 mL of filtrate was collected for immunoassay. In the recovery studies, the ground corn samples were spiked with FB_1 at five levels in 1 mL of water. These fortified samples were mixed thoroughly and incubated at room temperature for 30 min before extraction.

RESULTS AND DISCUSSION

Antibodies generated against FB_1 conjugates have resulted in distinctly different sensitivity and specificity depending on the choice of carrier proteins, adjuvants, and conjugation ratio, even though the same site of attachment and coupling chemistry were used (Usleber et al., 1994; Fukuda et al., 1994; Azcona-Olivera et al., 1992a). Among them, FB_1 conjugated to keyhole limpet hemocyanin (KLH) was reported to produce the most sensitive antibodies in rabbits with a detection limit of 10 ng/g in corn (Usleber et al., 1994). However, they used a much higher FB_1 to protein conjugation ratio, >700:1, than the well-established procedure of Azcona-Olivera which recommended a 50:1 ratio (Fukuda et al., 1994; Azcona-Olivera et al., 1992a).

In this investigation, we used the same conjugation ratio as Azcona-Olivera (1992a) for all FB_1 antigens. Antigens consisting of the amino group linked to HSA, using either Freund's adjuvants or CT as adjuvant (Lycke and Holmgren, 1986; Wilson et al., 1990), produced high-titer antibodies to bound FB_1 conjugate, but no inhibition of free FB_1 was observed. The apparent high titer might be due to the presence of antibodies that recognize the spacer arm between FB_1 and HSA or the conjugation byproducts. HSA by itself did not compete for the antibody binding either. The use of heterologous coating proteins such as FB_1-ovalbumin conjugate using a mixed anhydride method (Newsome et al., 1993) or the protected/deprotected FB_1-c-HSA did not change the outcome of the assay. Antibodies purified by protein A affinity chromatography, according to the method in the Pierce isolation and purification of IgG kit, did not improve their performance (Lindmark et al., 1983).

However, a sensitive ELISA could be developed from antibodies generated from FB_1-cholera toxin immunogen (Azcona-Olivera et al., 1992a) in rabbits with IC_{50} = to 0.3 ng/mL. Similar results were reported by Usleber et al. (1994) with IC_{50} and detection limit of 0.62 and 0.17 ng/mL, respectively. Unfortunately, this immunogen only produced low-titer antibodies. An antiserum concentration of 1:1000 gave an OD only to 0.3 during the optimization routine. Although the signal can be magnified 4-fold by using biotin and streptavidin complex (Yeung and Newsome, 1995), no further developmental work was pursued.

On the other hand, rabbits immunized with an antigen having an intact FB_1 primary amine, i.e., FB_1-c-HSA, produced high-affinity and avidity antibodies. Specific antibodies could be detected as early as 1 week after the first booster dose, and maximum responses occurred at 6−7 injections. Three out of four rabbits in this group gave similar high titers and IC_{50}. The fourth one produced about one-half the titer as its counterparts.

The ELISA developed from these antibodies had an IC_{50} of 0.66 ng of FB_1/mL and a limit of detection of 0.05 ng/mL of water with at least 10% binding inhibition. These data suggest the integrity of the amino group and/or the conformation of the FB_1 molecule is an important epitope for the FB_1 antibodies. Although it was not resolved which, or how many, carboxylic acids in FB_1 were coupled to the HSA, it is unlikely that two or more lysine residues are close together in HSA for multiple coupling. It was further anticipated, subsequent to deprotection of the FB_1 amine conjugate, that the amine would fold back to a cage conformation, since the three-dimensional molecular modeling of FB_1 showed the amino group folded back to the tricarballylic acid side chain in a cage configuration (Elissalde et al., 1995; Beier et al., 1995). In a structure−activity relationship study of fumonisins in carcinogenesis and cytotoxicity, Gelderblom and his associates have demonstrated that a free amino group was essential for the biological activity (Gelderblom, 1994). It is also obvious that the carballylic acid served as an effective spacer arm with a 6-atom chain length for the presentation of the hapten to the immune system of rabbits. However, it should be mentioned that in another study the same immunogen induced only very low titers in immunized goats (J. M. Yeung, Health Canada at Ottawa, unpublished results). Immunization for up to 12 months failed to boost the titer to a useful level.

Table 1. Specificity of Antisera toward Fumonisins, Related Compounds, and Other Mycotoxins

compound	IC_{50} (ng/mL)	cross-reactivity (%)
FB_1	0.658	100
FB_2	1.061	62
FB_3	4.634	14
HFB_1	6.380	11
HFB_2	22.527	3
tricarballylic acid	ND[a]	0
sphingosine	ND	0
sphinganine	ND	0
deoxynivalenol	ND	0
zearalenone	ND	0
T-2 toxin	ND	0
aflatoxin B_1	ND	0
ochratoxin A	ND	0

[a] No detectable inhibition at concentrations up to 500 ng/mL.

Common mycotoxins and fumonisins listed in Table 1 were tested for cross-reactivity by preparing standard curves in water, and their IC_{50} values were determined in the ELISA. The IC_{50} value, which is useful for comparing the degree of inhibition exhibited by various compounds, is defined as the concentration which inhibits the binding of the antibody to the plate by 50% when it is compared with the absence of the analyte. The cross-reactivity values were calculated as (IC_{50} of FB_1/IC_{50} of compound) × 100. Our anti-fumonisin antibodies only recognized the fumonisins and their hydrolyzed backbones but not other mycotoxins, such as aflatoxin B_1, ochratoxin A, deoxynivalenol, zearalenone, or T-2 toxin, commonly found in grains. Cross-reactivities (FB_1 = 100%) to FB_2, FB_3, HFB_1, and HFB_2 were 62%, 14%, 11%, and 3%, respectively. Furthermore, the antibodies did not cross-react with sphingolipids, which means this technique may be useful in detecting fumonisins in biological samples.

Since FB_1 has been found primarily in corn and corn-based products, an ELISA procedure was developed for corn. The procedure only required aqueous methanol extraction, filtration, and dilution before analysis. The linearity of the log−logit plot in the standard curve consistently gave $r^2 \geq 0.99$ in the range of 0.05−0.75 ng of FB_1/mL. No matrix effect on blank corn was observed at a detection limit of 5 ng/g. Corn samples spiked at five levels of FB_1 from 10 ng/g to 10 μg/g gave recoveries ranging from 76% to 92% with intra-assay coefficients of variation (cv) <10% (Table 2). Although similar results were obtained for the inter-assay cv for the procedure, which consisted of subjecting the sample matrix to the entire extraction and ELISA, somewhat larger cv's were observed at the lower concentrations (Table 3). The ruggedness of the assay was demonstrated in the small variability of the IC_{50} and the minimum detection limit derived from the standard

Table 2. Percent Recovery of FB_1 in Corn[a]

FB_1 added (ng/g)	mean ± SD	cv (%)	*n*
10	75.5 ± 4.2	5.5	4
50	82.0 ± 6.0	7.4	4
100	82.5 ± 8.1	9.8	4
1000	92.0 ± 6.1	6.6	4
10000	90.3 ± 7.2	8.0	4

[a] No quantifiable FB_1 was detected in blank corn, with the detection limit of 5 ng/g corn.

Table 3. Inter-assay Coefficients of Variation of the Procedure for FB_1 in Corn[a]

FB_1 added (ng/g)	mean ± SD	cv (%)	n
10	94.7 ± 16.8	17.8	6
50	90.0 ± 12.4	13.8	6
100	87.9 ± 5.0	5.7	6
1000	90.9 ± 7.7	8.5	6
10000	95.1 ± 6.9	7.3	6

[a] No quantifiable FB_1 was detected in blank corn, with the detection limit of 5 ng/g corn.

Table 4. Inter-assay Variability of IC_{50} and Minimum Detection Limit (MDL) during 1 Month[a]

	FB_1 (ng/mL) mean ± SD	cv (%)	n
IC_{50}	0.661 ± 0.056	8.5	12
MDL	0.049 ± 0.004	7.1	12

[a] Values are expressed in ng/mL FB_1 derived from a seven-points standard curve from each plate. MDL, minimum detection limit, is based on the lower datum point, which is at least 10% inhibition, in the standard curve.

curves. The cv's were 8.5% and 7.1%, respectively, over a 1 month period (Table 4). The procedure reported by Usleber et al. (1994) using a competitive direct EIA had a detection limit for FB_1 of 0.17 ng/mL of buffer and about 10 ng/g in corn and recoveries of 60% and 73% at levels of 50 and 500 ng/g, respectively. The present assay appears to offer increased sensitivity, recovery, and reproducibility than any previously developed fumonisin immunoassays, thus far.

In summary, we report here an alternative method of coupling an amphoteric compound, FB_1, to a carrier protein through a selective attachment of a carboxylic acid moiety by incorporating protecting and deprotecting steps for the amine. The antibodies generated were highly specific to fumonisins and their aminopolyol hydrolysis products. The sensitive ELISA procedure developed for corn does not require sample cleanup. The use of this assay for routine screening or monitoring of FB_1 in corn-based products can be envisioned. A comparative ELISA and HPLC study in a survey of corn-based products is underway.

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