Enzyme Immunoassay for Fumonisin B_1 Applied to Corn-Based Food

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Polyclonal antibodies against fumonisin B_1 (FB_1) were produced in rabbits after immunization with a FB_1-keyhole limpet hemocyanin conjugate. Using these antibodies and a FB_1-horseradish peroxidase conjugate in competitive direct enzyme immunoassay (EIA), the average detection limit for FB_1 (buffer solutions) was 0.17 ng/mL. The relative cross-reactions with FB_1, fumonisin B_2, and fumonisin B_3 were 100%, 24%, and 55%, respectively. Corn samples were extracted with methanol/water and the diluted raw extracts directly analyzed by EIA. The raw extracts were purified by employing strong ion-exchange (SAX) cartridges and reanalyzed by both EIA and a HPLC method. The recoveries for FB_1 from artificially contaminated corn at levels of 50 and 500 ng/g were 59.7% and 73.2% after EIA analysis of the raw extracts; the corresponding values for SAX extracts were 53.5% and 54.4% (EIA) and 62.3% and 65.6% (HPLC), respectively. Food samples ($n = 19$) from German retail stores (some were import products from Italy) were analyzed: 16 were found to contain FB_1 in a range from 12 to 1300 ng/g.

INTRODUCTION

Fumonisins (Figure 1) are a group of mycotoxins discovered in 1988 from cultures of *Fusarium moniliforme* (Gelderblom et al., 1988) and since have shown to be of high importance as natural occurring contaminants in corn worldwide [for reviews see Norred (1993) and Scott (1993)]. The most abundant of these, fumonisin B_1 (FB_1), was found to induce leukoencephalomalacia in horse (Marasas et al., 1988), pulmonary edema in swine (Colvin and Harrison, 1992; Haschek et al., 1992), and hepatic cancer in rats [Gelderblom et al., 1988; for a review see also, Ross et al. 1992)]. A possible role of high levels of fumonisins in corn in the etiology of human esophageal cancer is under discussion (Sydenham et al., 1990).

The known toxic effects, as well as the naturally occurring levels of fumonisins in corn, present a potential threat to human and animal health (Thiel et al., 1992), which suggests the need for screening and analytical methods to routinely monitor for the presence of fumonisins in foods and feeds. The currently most frequently used analytical methods for fumonisins are high-performance liquid chromatographic (HPLC) systems [e.g., Shephard et al. (1990), Scott and Lawrence (1992), and Stack and Eppley (1992)], but thin-layer chromatography (Gelderblom et al., 1988), gas chromatography (Sydenham et al., 1990), and HPLC/mass spectrometry (Young and Lafontaine, 1993) have also been described. Costs and time requirements, especially for sample cleanup steps, make most of these methods unsuitable to process large numbers of samples.

In such a situation, immunochemical approaches have been shown to be good alternatives for the screening of cereals for mycotoxins (Pestka, 1988; Chu, 1990). So far (November 1993) only one (monoclonal antibody-based) enzyme immunoassay has been described for fumonisins (Azcona-Olivera et al., 1992a) with a detection limit for FB_1 in corn in the parts per million (ppm) range. A commercial test (FumoniTest, Vicam, Watertown, MA) uses monoclonal antibodies in an affinity column for fumonisin detection at the 1 ppm level. The objective of this study was to develop an enzyme immunoassay which should enable FB_1 detection in corn for human consumption down to the low parts per billion (ppb) level, a concentration range that is currently the optimum for most liquid chromatographic methods. A simple extraction method was tested and applied to corn-based food samples. The results of this rapid method were compared to those obtained by a HPLC method which is essentially based on a method originally described by Shepard et al. (1990), improved by Sydenham et al. (1992), and successfully used in an IUPAC collaborative study (Thiel et al., 1993).

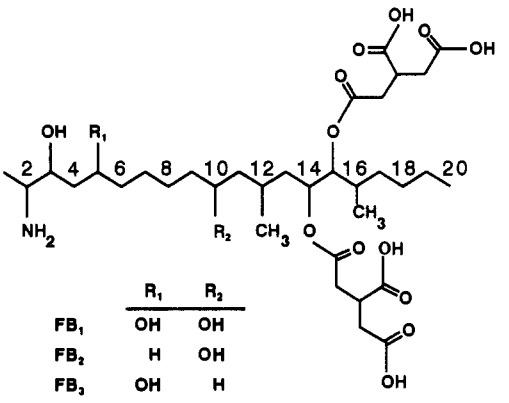

Figure 1. Structures of fumonisins B_1, B_2, and B_3 [according to Plattner et al. (1992)].

EXPERIMENTAL PROCEDURES

Materials. Safety Note: Fumonisins are suspected carcinogens and should be handled with care.

FB_1 was purchased from Sigma Chemicals, Deisenhofen, Germany. Fumonisin B_2 (FB_2), prepared as described by Voss et al. (1993), was a gift from William J. Chamberlain, Richard B. Russell Agricultural Research Center, U. S. Department of Agriculture, Athens, GA. Fumonisin B_3 (FB_3) was obtained from PROMEC, Tygerberg, South Africa. Keyhole limpet hemocyanin was from Boehringer, Mannheim, Germany. Horseradish peroxidase (HRP), glutaraldehyde, sodium periodate, and all other chemical used in this study were from Sigma. All chemicals and solvents used were of at least analytical grade: methanol was of HPLC grade.

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Preparation of a FB_1–Horseradish Peroxidase Conjugate. The periodate method (Wilson and Nakane, 1978) was used for coupling of FB_1 to HRP. The HRP (4 mg in 1 mL of distilled water) was activated by reaction with sodium periodate (4.2 mg in 0.2 mL of distilled water) for 20 min at room temperature and then dialyzed against 1 mM sodium acetate buffer (pH 4.4; 2 × 5 L) for 12 h. An aliquot of the activated HRP (2.8 mg) was mixed with 1 mL of an aqueous FB_1 solution (1 mg), the pH was adjusted to approximately 9.5 with 0.1 M NaOH, and the reaction mixture was incubated for 2 h at room temperature. Then 0.1 mL of a sodium borohydride solution (4 mg/mL distilled water) was added and incubated for 1 h at 4 °C, and finally the conjugate was dialyzed against phosphate-buffered saline (PBS; 0.15 M, pH 7.2, 3 × 5 L), lyophilized in small portions, and stored at −20 °C. The peroxidase concentration was determined from the absorbance at 403 nm.

Preparation of the Immunogen. FB_1 was coupled to keyhole limpet hemocyanin (KLH) via glutaraldehyde reaction (Avrameas and Ternynck, 1969). KLH (10 mg) was dissolved in 1 mL of PBS and dialyzed overnight against PBS (1 × 5 L). FB_1 (1 mg) was dissolved with an aliquot (420 μL) of the dialyzed KLH solution, glutaraldehyde solution (1% in water; 14 μL) was added, and the mixture was incubated for 3 h at room temperature. The conjugate was dialyzed against PBS (3 × 5 L), lyophilized, and stored frozen at −20 °C. The protein concentration was determined from the absorbance at 280 nm according to the manufacturer's instructions.

Immunization. Three rabbits (female chinchilla bastard; Savo Ivanova, Kisslegg, Germany) were each immunized by multiple intracutaneous injections of an emulsion of water (0.5 mL) and complete Freund's adjuvant (1.5 mL) containing the FB_1–KLH (250 μg). Blood samples were collected in 2-week intervals, and the antibody titers were monitored using the FB_1–HRP in a double-antibody solid-phase enzyme immunoassay technique as described earlier (Märtlbauer et al., 1988). Subcutaneous booster injections using the same immunogen composition were given individually as soon as the relative antibody titer decreased. Serum was precipitated with ammonium sulfate (70%, in distilled water) according to the method of Hebert et al. (1973), dialyzed against PBS, and stored frozen at −20 °C.

Competitive Direct EIA. The wells of a microtiter plate were coated overnight at room temperature with 100 μL of anti-FB_1 antiserum, diluted 1:1500 with 0.1 M sodium carbonate/bicarbonate buffer (pH 9.6). Free protein binding sites of the wells were blocked with 200 μL of 2% sodium caseinate in PBS for 30 min at room temperature, and then the plate was washed and drained. Next, 50 μL of FB_1 standard solution (in 10% methanol/PBS) was simultaneously incubated with 50 μL of FB_1–HRP (in 1% sodium caseinate/PBS; 25 ng/mL) for 2 h at room temperature. The plate was washed, and substrate solution (1 mM 3,3′,5,5′-tetramethylbenzidine and 3 mM H_2O_2 per liter of potassium citrate buffer, pH 3.9; Gallati and Pracht, 1985) was added (100 μL/well). After 15 min, the reaction was stopped with 1 M H_2SO_4 (100 μL/well), and the absorbance at 450 nm was measured (400 AT microplate reader, SLT, Crailsheim, Germany). Throughout the study, all standard and sample determinations were performed in quadruplicate. The toxin content was quantified using an on-line PC and EIA software developed by Märtlbauer (1993), which uses a cubic spline function for calculation of the standard curve. The program also calculates the detection limit (Student's t, n = 4; 95% confidence limit) and the 50% inhibition dose. The measuring range of the standard curve usually is from 20% to 80% relative binding ($B/B_0 \times 100$). To determine the test specificity, fumonisins B_1, B_2, and B_3 were tested for competition with the FB_1–HRP under the conditions of the EIA, whereby the three serial toxin standard dilutions were performed in the same microtiter plate. The absorbance values were plotted point-to-point by a submenu of the software, and the relative cross-reactivity of each toxin was calculated on the basis of the concentration necessary to inhibit 50% FB_1–HRP binding. Typical results for FB_1, FB_2, and FB_3 by EIA are shown in Figure 2.

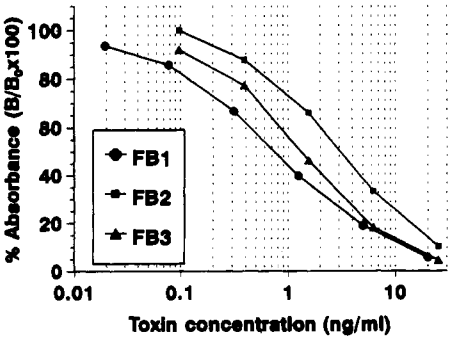

Figure 2. Competitive direct EIA standard curves for fumonisins B_1, B_2, and B_3. The x-axis (log scale) indicates the toxin concentration. The y-axis indicates the relative binding of FB_1–HRP (B/B_0), expressed as (absorbance units corresponding to a standard toxin concentration/absorbance of toxin negative buffer solution) × 100. B_0 was 1.1 absorbance units. Each point represents the mean of four replicates. The coefficients of variation were between 1.2% and 7.3%.

HPLC. A method originally described by Shepard et al. (1990) and improved by Sydenham et al. (1992) was used for HPLC detection of FB_1 with some modifications. A Merck LichroCart 125–4 column, filled with LiChrospher 100 RP-8 material, was used as the stationary phase. The mobile phase was methanol (669 mL)/0.1 M NaH_2PO_4 (340 mL), adjusted to a pH of 3.4 with orthophosphoric acid, in a flow rate of 1 mL/min. For toxin derivatization, 50 μL of standard FB_1 (in methanol) was mixed with 200 μL of o-phthaldialdehyde (OPA) solution (Sydenham et al., 1992), and then 20 μL was injected into the HPLC system using a Waters Model 712 WISP injector. Derivatized FB_1 (retention time 12.8 min) was detected using a Shimadzu RF 535 fluorescence detector (excitation at 335 nm; emission at 440 nm). Toxin concentrations (injection range from 2 to 40 ng) were calculated from peak areas using Nelson EG software (LKB, Bromma, Sweden).

Corn Sample Extraction. Ground corn samples (25 g) were extracted with 50 mL of methanol/water (75/25) for 20 min on a magnetic stirrer. The extract was centrifuged and the supernatant filtered through a paper filter. An aliquot of this raw extract was adjusted to a methanol content of 10% with PBS, further diluted at least 1:3 in 10% methanol/PBS, and directly assayed by EIA. An aliquot of the raw extract (10 mL) was further purified using strong anion exchange (SAX) cartridges [Adsorbex (Merck); 400 mg per cartridge] according to the detailed instructions given by Sydenham et al. (1992). The final volume of the purified and concentrated extract was 0.2 mL of methanol, corresponding to 5 g of sample. A small portion of this SAX extract was diluted 1:10 with PBS, further diluted with 10% methanol/PBS, and analyzed by EIA. For the determination of FB_1 by HPLC, 50 μL of the purified extract was added to 200 μL of OPA

solution and further treated as described for toxin standards. Recovery (EIA, HPLC) of FB_1 from artificially contaminated corn samples was studied at concentrations of 50 and 500 ng/g, respectively. Food samples (popcorn; corn grit; corn semolina, in Germany known as polenta) were purchased from local retail stores from October 1992 to March 1993 and analyzed by EIA and HPLC. Five of these samples were import products from Italy.

RESULTS AND DISCUSSION

Antibodies could be detected in the sera of all rabbits immunized with the FB_1–KLH conjugate as early as 4 weeks after the initial exposure; high relative serum titers (>1:100000) were obtained after the first booster. The use of KLH (molecular weight 3 000 000– 7 500 000) as a protein carrier, together with well-established standard conjugation procedures [used also by Azcona-Olivera et al. (1992b)] for the production of the FB_1 immunogens, seems to be promising if other carriers fail to induce sufficient immune response.

The serum that showed the highest affinity for FB_1 as determined by direct EIA was used for the establishment of the immunoassay. The intraassay (4-fold determinations) and interassay coefficients of variation were usually below 7.5% and 14%, respectively. After evaluation of 58 standard curves performed within a period of 6 months, the mean detection limit and 50% dose were found at 167 and 623 pg/mL, respectively. When specificity was checked under the conditions of the EIA, the relative cross-reactivities with FB_1, FB_2, and FB_3 were found to be 100%, 24%, and 55%, respectively. The detection limits for FB_2 and FB_3 were found to be at 1200 and 460 pg/mL, respectively. With these test characteristics, the EIA described here is about 2 orders of magnitude more sensitive than an monoclonal antibody-based EIA described by Azcona-Olivera et al. (1992a).

Due to the high sensitivity of the assay, a simplified sample preparation procedure, omitting the expensive and time-consuming cleanup with SAX cartridges, was sufficent for EIA analysis, resulting in a detection limit for FB_1 in corn of about 10 ng/g. To check the data obtained by EIA using the rapid extraction procedure, the raw extracts were additionally analyzed by HPLC and EIA after SAX purification. As the SAX cleanup includes a 25-fold concentration step of the sample, the theoretically achievable detection limit of the EIA (167 pg/mL buffer) in corn would then be at less than 10 pg of FB_1/g for purified extracts. When corn SAX extracts were redissolved in 10% methanol/PBS and assayed by EIA, almost 100% of the samples were found to be contaminated with FB_1 in the picograms per gram range (data not shown). However, as no method was available to confirm results in this low concentration range, for this study the detection limit was adjusted to 10 ng/g by diluting SAX extracts with buffer. The recoveries for FB_1 at the 50 and 500 ng/g level, as determined by the three analyzes, are listed in Table 1. In general, the data show overall agreement between both sample preparation procedures. However, the average recoveries were lower than those reported by others, and a tendency of higher EIA values for raw extracts than for SAX extracts was observed.

Various procedures have been proposed for initial extraction of fumonisins from contaminated corn samples; most of them employ methanol/water (3 + 1) as the extraction solvent. According to Sydenham et al. (1992), the best recoveries for fumonisins from contaminated corn could be achieved with a Polytron homogenizer (1–5 min) or a Sorvall Omnimixer (5 min) for initial sample extraction, although no recoveries were given for the entire procedure. However, Stack and Eppley (1992), who used a Waring Model EP-1 blender at high speed for 2 min, obtained average recoveries of only 67% for FB_1. According to Sydenham et al. (1992), extraction for 60 min on a wrist-action shaker gave low recoveries. On the contrary, Scott and Lawrence (1992) obtained recovery rates for FB_1 of 94% after shaking for 15–30 min. Thus, both extraction time and blending intensity seem to influence the recoveries of fumonisins. In our experiments, magnetic stirring of the sample–solvent mixture for 20 min was not very efficient (recovery 59–73% for raw extracts) but gave consistent results within the concentration range of interest. It also was very convenient for the simultaneous extraction of more than 10 samples, an important aspect for a large-scale screening method.

Table 1. Comparison of the Recovery of Fumonisin B_1 from Artificially Contaminated Corn Samples Using SAX-Purified Extracts (HPLC, EIA) and Raw Methanolic Extracts (EIA)

FB_1 added (ng/g)		FB_1 found (ng/g) HPLC (SAX)	EIA (SAX)	EIA (raw extract)
0		<10	<10	<10
50		37.4	32.9	33.6
50		27.2	23.4	25.3
50		25.0	26.8	28.3
50		35.9	22.8	31.8
50		30.2	27.9	30.2
	mean ± SD	31.1 ± 5.4	26.8 ± 4.1	29.8 ± 3.2
	recovery (%)	62.3	53.5	59.7
500		335	246	320
500		270	238	360
500		379	340	418
500		358	260	349
500		296	274	381
	mean ± SD	328 ± 44.5	272 ± 40.6	366 ± 36.6
	recovery (%)	65.6	54.4	73.2

Azcona-Olivera et al. (1992a) used acetonitrile/water (1 + 1) and extraction of fumonisins by shaking for 45 min. Diluted raw extracts corresponding to 50 mg of corn/mL were directly analyzed by their EIA method. Recoveries for fumonisin B_1 at concentration levels of 5, 10, and 25 μg/g were 117–168%, 86–114%, and 58–83%, respectively. In our own experiments, initial tests showed that a 50 mg of corn/mL extract may result in unspecific binding inhibition of 20–25% in the EIA. This particularly affected the recovery for low FB_1 concentrations (10–20 ng/g) near the detection limit, as this unspecific binding inhibition adds to the specific binding inhibition by the toxin. Thus, virtual recoveries of 100% and higher are obtained. For higher toxin concentrations (20–50 ng/g), this unspecific sample matrix interference was less obvious, but still detectable, since the toxin values did not fit together with those obtained for the same extract in a higher dilution. Several tests, using serial extract dilutions of the few corn samples that were found to be almost FB_1-negative by both EIA and HPLC, showed that a minimum dilution of corn extracts equivalent to 20 mg/mL of corn was necessary to give absorbance values >95% of the negative buffer control solution.

The EIA values for raw extracts, especially of naturally contaminated samples (Table 2), were somewhat higher than those obtained for SAX extracts. Considering the EIA and HPLC results for artificially contaminated samples, this is only in part due to a loss of FB_1 during

Table 2. Comparison of the FB_1 Values in Naturally Contaminated Corn Samples Determined in Purified Extracts by HPLC and EIA and in Raw Extracts by EIA

	SAX-purified extract		
sample	HPLC value[a] (ng/g)	EIA value[b] (ng/g)	raw extract EIA value[b] (ng/g)
popcorn D[c]	<10	<10	<10
popcorn D	<10	<10	20.8
popcorn D	12.3	17.6	35.4
popcorn D	46.8	46.6	51.6
popcorn D	114	122	175
popcorn D	104	118	146
corn grit D	<10	<10	<10
corn grit D	13.9	20.8	58.9
semolina D	19.9	16.0	26.9
semolina D	17.3	23.2	63.3
semolina D	20.7	24.7	32.8
semolina D	27.5	33.1	60.6
semolina D	<10	<10	<10
semolina I[d]	364	231	354
semolina I	520	674	679
semolina I	45.6	75.7	284
semolina I	1230	984	1280
semolina I	650	724	1000
semolina I	737	1120	889

[a] Single determinations. [b] Serial dilutions (4-fold determinations) of the extracts were analyzed. The dilution that gave absorbance values closest to the 50% dose (40–60%) was used for calculation. [c,d] Presumable country of origin: [c]D, Germany; [d]I, Italy.

the cleanup step. At least for the lower FB_1 values (<100 ng/g), it cannot be excluded that some samples had a higher matrix effect which led to an overestimation of the toxin content as aforementioned. For higher toxin concentrations, however, the sample extracts were diluted 1:1000 and higher for EIA analysis, thus eliminating unspecific effects. As fumonisins B_2 and B_3 were not determined by HPLC, no data are available as to whether or not these toxins were present in some samples. As the EIA strongly cross-reacts with these toxins, and maybe with other (unknown) fumonisins, this could also give higher EIA values for raw extracts, provided their recovery from SAX cartridges is low.

Another possibility should be taken into consideration, which is deduced from the findings of Plattner et al. (1992). These authors observed a pH-dependent equilibrium between the open chain and a cyclic structure for the propane-1,2,3-tricarboxylate ester (Figure 1). As the elution of FB_1 from the SAX cartridges is achieved using 1% acetic acid in methanol, the ratio of the two structures may be different in raw extracts (methanol/water) and SAX extracts. The propane-1,2,3-tricarboxylate ester side chains are important for antibody binding (Azcona-Olivera et al., 1992a); therefore, this may contribute to the observed differences between both sample preparations. However, further studies are required to examine whether such an effect can be demonstrated.

The analysis of food samples (n = 19) from Munich retail stores showed that corn meal and popcorn both were frequently (84%) contaminated with FB_1, although most samples had toxin levels of less than 100 ng/g. Maximum values in corn semolina exceeded 1 μg/g FB_1. Only one sample, which had 20 ng of FB_1/g after analysis of the raw extract, was negative after SAX cleanup; three samples that were negative in the rapid test were also negative by EIA and HPLC after extract purification. The overall agreement for the positive samples between HPLC (x) and EIA (y) (purified extracts: $y = 0.991x + 22.8$; $r = 0.939$; raw extracts: $y = 1.10x + 54.8$, $r = 0.974$) was acceptable. It is interesting to note that all samples exceeding FB_1 values of 200 ng/g were imported corn semolina from Italy. Due to the common market within the European Union, however, corn producers and trading companies are not necessarily from the same country. In general, the FB_1 concentrations found in food samples were in the same range as reported from, e.g., Switzerland (Pittet et al., 1992), the United States (Hopmans and Murphy, 1993; Stack and Eppley, 1993), Asia (Ueno et al., 1993), and various other countries (Sydenham et al., 1991).

In conclusion, we have developed an immunochemical test for FB_1 that is both sensitive and easy to perform. The assay could be used as a screening method for FB_1 in corn and corn-based products, as large numbers of samples could be analyzed without time-consuming sample extract treatment. The frequent contamination of corn for human consumption as found in this study shows the need to routinely screen corn-based foods for this toxin. Further development of a dipstick assay format to provide a rapid immunochemical test (Schneider et al., 1991) for FB_1 is under study.

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