Development of a Polyclonal Antibody-Based Sensitive Enzyme-Linked Immunosorbent Assay for Fumonisin B_4

Hanne R. Christensen,[†] Feng-Yih Yu,[‡] and Fun S. Chu*,[†]

Food Research Institute and Department of Food Microbiology and Toxicology, University of Wisconsin−Madison, Madison, Wisconsin 53706, and Centre of Advanced Food Research, Technical University of Denmark, DK-2800 Lyngby, Denmark

Polyclonal antibodies (PAb) against fumonisin B_4 (FmB_4), which have good cross-reactivity with four major fumonisins, were produced by immunizing a rabbit with FmB_4−keyhole limpet hemocyanin conjugate. A sensitive competitive direct enzyme-linked immunosorbent assay (CD-ELISA) for fumonisins was developed. Because of the limited supply of FmB_4, both FmB_1−horseradish peroxidase conjugate (HRP) and FmB_3−HRP were tested as the toxin-enzyme markers in the CD-ELISA. In the FmB_1−HRP-based CD-ELISA, the concentrations of FmB_1, FmB_2, FmB_3, and FmB_4 causing 50% inhibition of binding of enzyme marker (IC_{50}) were 9.0, 2.1, 9.0, and 6.5 ng/mL (or the relative cross-reactivities toward FmB_1, FmB_2, FmB_3, and FmB_4 were 58.5, 309.5, 58.5, and 100%), respectively. In the FmB_3−HRP-based CD-ELISA, the IC_{50} values for FmB_1, FmB_2, FmB_3, and FmB_4 were 7.1, 1.9, 7.6, and 5.3 ng/mL (or the relative cross-reactivities toward FmB_1, FmB_2, FmB_3, and FmB_4 were 74, 280, 70, and 100%), respectively. The FmB_3−HRP-based CD-ELISA was then used in a series of analytical recovery experiments using *Fusarium moniliforme* corn culture material spiked with FmB_1 and with clean corn spiked with a FmB_3/FmB_4-containing extract. The overall recovery of FmB_1 from culture material in the range of 10−100 ppm was 65%. The detection limit for FmB_1 with clean corn as matrix was between 100 and 500 ppb. *F. moniliforme* cultures were analyzed with the developed CD-ELISA and a well-established FmB_1 antibody-based ELISA, which is not sensitive to FmB_4. Differences in the fumonisin levels found by the two assays were used as an indication of the presence of FmB_4 in the culture material and, therefore, as a method to identify FmB_4-producing strains. Using ELISA in combination with HPLC individual B-series fumonisins were quantified. The ELISA developed in the present study would be a useful supplement to FmB_1 antibody-based ELISA for screening of *Fusarium* strains for the production of major fumonisins.

Keywords: *ELISA; fumonisins; FmB_1; FmB_4; mycotoxins; polyclonal antibodies*

INTRODUCTION

Fumonisins (Fm) are a group of mycotoxins primarily produced by *Fusarium moniliforme*, one of the most common fungi colonizing corn throughout the world (Dutton, 1996; Jackson et al., 1996; Nelson et al., 1991, 1992; Norred, 1993; Riley and Richard, 1992). More than 11 structurally related Fms (B_1, B_2, B_3, B_4, C_1, C_4, A_1, A_2, etc.) have been found since the discovery of FmB_1 in 1988 (Gelderblom et al., 1988). They are most frequently found in corn, corn-based foods, and other grains (such as sorghum and rice), and the level of contamination varies considerably with different regions and years (range from 0 to >100 ppm, generally <1 ppm). The toxicities of *F. moniliforme*-contaminated corn as well as pure fumonisins have been studied extensively and have been found to cause various species-specific diseases in animals, including equine leukoencephalomalacia (ELEM) (Kellerman et al., 1990; Marasas et al., 1988) and porcine pulmonary edema (PPE) (Colvin and Harrison, 1992; Harrison et al., 1990; Osweiler et al., 1992). Although FmB_1 was originally found to be a potent cancer promoter (Gelderblom et al., 1991) and considered as a Class 2B carcinogen in rats, subsequent studies showed that all three major Fms, that is, FmB_1, FmB_2, and FmB_3, are carcinogens; all of them have cancer initiation and promoting activities in rats (IARC, 1993). Kidney has also been found as a target organ of this carcinogen (Bucci et al., 1998). Several studies have also indicated that human consumption of fumonisin-contaminated corn may be associated with esophageal cancer (Rheeder et al., 1992; Sydenham et al., 1990; Thiel et al., 1992).

Of the currently identified fumonisins, fumonisin B_1 and to much lesser extents FmB_2 and FmB_3 are the most abundant fumonisins occurring naturally (Dutton, 1996; Murphy et al., 1993; Shephard et al., 1996). To avoid human and animal consumption of the contaminated foods and feed, extensive studies for the development of sensitive methods for the detection of this group of mycotoxins have been made (Trucksess, 1998, 1999; Wilson et al., 1998). Sensitive chemical and immunochemical methods are now available. However, whereas immunochemical methods can achieve the objective of both sensitivity and simplicity (rapid screening), antibodies used in these assay are primarily reactive with FmB_1, FmB_2, and FmB_3 (Chu, 1996a,b). For example, antibodies generated against FmB_1 in our

* Author to whom correspondence should be addressed [fax (608) 263-1114; e-mail fschu@facstaff.wisc.edu].

[†] Technical University of Denmark.

[‡] University of Wisconsin−Madison.

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laboratory generally also show relatively high cross-reactivities with FmB_2 and FmB_3 (Yu and Chu, 1996, 1999a,b; Chu, 1996b). Most antibodies used for ELISA of fumonisin do not react with the rare fumonisins, including FmB_4, which have been proposed as likely key precursors for FmB_1 biosynthesis (Desjardins et al., 1996). Thus, there is a need to generate specific antibodies against FmB_4 for use in an ELISA that can rapidly detect this fumonisin in *Fusarium* cultures/mutants involved in the fumonisin biosynthetic studies. With such a need, the present study was conducted. Our objectives were (a) to produce high-affinity polyclonal antibodies (PAb) against FmB_4, (b) to use these antibodies to establish a sensitive competitive direct ELISA (CD-ELISA) protocol, and (c) to test the CD-ELISA in combination with HPLC for individual determination of FmB_4 in addition to FmB_{1-3} in the *Fusarium* culture material. Details for the production, purification, and characterization of the antibodies as well as the establishment and examples of use of the ELISA are described herein. This is the first time that production of antibodies against FmB_4 is reported.

MATERIALS AND METHODS

Materials. *Reagents.* Fumonisin B_1 was kindly provided by Dr. Robert M. Eppley of the Food and Drug Administration (FDA) (Washington, DC). Fumonisin B_4 was kindly supplied by Dr. R. D. Plattner and FmB_2 and FmB_3 were supplied by Dr. Chris M. Maragos, both at the National Center for Agricultural Utilization Research (NCAUR), U.S. Department of Agriculture (USDA) (Peoria, IL). Keyhole limpet hemocyanin (KLH), horseradish peroxidase (HRP; ELISA grade), and goat anti-rabbit IgG−HRP conjugate were obtained from Pierce Chemical Co. (Rockford, IL). Water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EDC] was obtained from Aldrich Chemical Co. (Milwaukee, WI). *Pasteurella*-negative New Zealand white rabbit was purchased from LSR Industries (Union Grove, WI). Freund's complete adjuvant containing *Mycobacterium smegmatis*/L and Freund's incomplete adjuvant were purchased from Life Technologies (Grand Island, NY). Bovine serum albumin (BSA), ovalbumin (OVA), glutaraldehyde (25% aqueous solution, grade I), gelatin, L-lysine, and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). 3,3′,5,5′-Tetramethylbenzidine (K-Blue) was purchased from ELISA Technologies (Lexington, KY). Preswollen diethylaminoethyl cellulose anion exchanger (DE52) was obtained from Whatman International (Maidstone, U.K.). Methanol used for HPLC was of HPLC grade. All other chemicals and solvents used were of reagent grade or better.

Buffers. Phosphate-buffered saline (PBS), pH 7.4, was a 0.01 M sodium phosphate buffer containing 0.15 mol of NaCl/L. PB buffer was made as PBS but without NaCl. Washing buffer (PBS-T) consisted of 0.05% (v/v) Tween 20 in PBS. Conjugation buffer (CB), pH 4.7, consisted of 0.1 M MES [2-(*N*-morpholino)-ethanesulfonic acid], 0.9 M NaCl, and 0.02% NaN_3. Carbonate−bicarbonate buffer, pH 9.5, contained 0.022 M anhydrous sodium carbonate and 0.028 M sodium bicarbonate. Tris buffer (starting buffer), pH 8.5, contained 0.01 M tris(hydroxymethyl)aminomethane.

Materials and Instruments. Microtiter plates (Nunc Maxisorp modules 469914 and Nunc plates 269620; Nunc, Roskilde, Denmark) were washed with PBS-T with four wash and soak cycles. Absorbance (*A*) for solutions in microtiter wells was measured on a Thermo-max microplate reader (Molecular Devices Co., Menlo Park, CA) at 450 nm with 650 nm as reference. A Pharmacia GM-1 gradient mixer (Pharmacia, Uppsala, Sweden) was used to make the linear gradient in ion exchange chromatography. Absorbance of protein solutions was measured on a Beckman DU-7 spectrophotometer at 280 nm (1.0-cm path length) using an extinction coefficient of 1 mL mg^{-1} cm^{-1}. The HPLC system consisted of a reverse phase ODS column (4.6 × 250 mm, 5 μm sphereclones) from Phenomenex (Torrance, CA) and a Beckman System Gold 125 solvent module controlled by a Gold Nouveau chromatography data system (version 1.5) (Beckman Instruments, Schaumburg, IL).

Preparation of Various Fumonisin Conjugates. *Conjugation of FmB_4 to KLH.* FmB_4 was conjugated to KLH via the water soluble carbodiimide (WSC) method described previously (Yu and Chu, 1996). Briefly, 1 mg of FmB_4 in 0.5 mL of CB was mixed with 2 mg of KHL in 0.4 mL of deionized water to which 0.1 mL of a freshly prepared EDC solution (10 mg/mL deionized water) was slowly added. Two hours after incubation at room temperature, the reaction mixture was dialyzed against 2 L of PBS buffer at 4 °C for 72 h with two changes of buffer.

Conjugation of FmB_3 to HRP. Conjugation of FmB_3 to HRP was also made via the WSC method (Yu and Chu, 1996). In this reaction, 0.2 mg of FmB_3 in 0.4 mL of 25% (v/v) ethanol was mixed with 4 mg of EDC to which 0.5 mg of HRP in 0.5 mL of 25% (v/v) ethanol was added dropwise with stirring and another 4 mg of EDC was added. Twenty-five minutes after reaction at room temperature, another 4 mg of EDC was added. The mixture was kept at 4 °C overnight with constant stirring and followed by dialysis against 2 L of PBS for 72 h with two changes of buffer. The FmB_3−HRP was mixed 1:1 with glycerol and stored at −20 °C. Likewise, FmB_1−HRP was prepared.

Conjugation of FmB_3 to Ovalbumin (OVA). FmB_3 was cross-linked to OVA with glutaraldehyde via a two-step method of Avrameas and Ternynck (1969). OVA was first activated with glutaraldehyde as follows: to 5 mg of OVA in 0.2 mL of PB was added 10 μL of glutaraldehyde. After incubation at room temperature with constant stirring overnight, the mixture was dialyzed against 2 L of PBS overnight at 4 °C. For cross-linking, a solution containing 0.5 mg of FmB_3 in 0.1 mL of 50% (v/v) of ethanol plus 0.1 mL of distilled water was added to a half volume of the activated OVA dialysate (∼0.15 mL, 2.5 mg of OVA), followed by the addition of 0.1 mL of 1 M carbonate−bicarbonate buffer (pH 9.5). After incubation at 4 °C overnight with constant stirring, the reaction was stopped by the addition of 0.05 mL of 1 M lysine, pH 7 (0.1 M final) and allowed to stand at 4 °C with constant stirring for 2.5 h. The conjugate preparation was dialyzed against 2 L for 72 h with two changes of buffer and then lyophilized and stored at −20 °C. Likewise, FmB_1−OVA conjugate was prepared.

Production and Purification of Polyclonal Antibody. *Immunization.* For immunization, the schedule and methods of injection described by Chu and Ueno (1977) were used. In the initial immunization, 0.5 mg of FmB_4−KLH conjugate in 0.7 mL of PBS was emulsified with 0.7 mL of Freund's complete adjuvant (FCA) and then injected intradermally at 20 sites of the shaved back of a rabbit. Booster injections consisting of the same amount of immunogen but emulsified with Freund's incomplete adjuvant (FIA) were given at 2 sites on the back of the rabbit every 4−6 weeks. Trial bleedings via the marginal ear vein were begun 5 weeks after initial injection.

Partial Purification of Antiserum with $(NH_4)_2SO_4$. The collected antiserum was precipitated twice with $(NH_4)_2SO_4$ to a final saturation of 35% using a 100% saturated $(NH_4)_2SO_4$ solution (Hsu and Chu, 1994). The precipitate was redissolved in distilled water equal to half of the original volume and then dialyzed against 2 L of PBS overnight at 4 °C.

Further Purification of the Antibody by Ion Exchange Chromatography. Antiserum from the 12th week's bleed was further purified by ion exchange chromatography with a linear gradient of increasing ion strength of the elution buffer (Harlow and Lane, 1988). In a typical run, 10 mL (210 mg of protein) of the ammonium sulfate-purified antiserum was dialyzed against 2 L of 0.01 M Tris buffer (starting buffer) overnight at 4 °C and then applied on a DEAE-cellulose (DE52) column (13 × 1.6 cm; 26 mL) that had been previously equilibrated with starting buffer. After washing with 150 mL of starting buffer, the proteins were eluted sequentially with a gradient of increasing concentration of NaCl (0−0.3 M; 2 ×

250 mL) in Tris buffer using a gradient mixer. The flow rate was 0.5 mL/min, and fractions of 4 mL per tube were collected. Absorbance at 280 nm of each fraction was measured, and an elution curve was drawn. Representative fractions were tested with the CD-ELISA as described below. Fractions with appropriate response of high displacement by FmB_4 were pooled and stored at −20 °C.

Monitoring of Antibody Titers by Indirect ELISA. The antibody titers were determined by an indirect ELISA using FmB_3−OVA conjugate as the solid-phase antigen (Chu et al., 1995). Microtiter plate wells were each coated with 0.1 mL of FmB_3−OVA conjugate (diluted 1:10000; 0.5 μg/mL) overnight at 4 °C. After three washes with washing buffer (3×), the wells were blocked for nonspecific binding with 0.18 mL of 0.1% gelatin in PBS at 37 °C for 30 min. The plate was washed again, and 0.1 mL of serially diluted antiserum was added to each well. After incubation at 37 °C for 1 h, the unbound antibody was removed by washing and 0.1 mL of goat anti-rabbit IgG−HRP conjugate (1:10000) was added. After 45 min of incubation at 37 °C, the plate was washed again, and 0.1 mL of K-blue substrate was added to each well. The reaction was stopped by adding 0.1 mL/well of 1 M HCl after development of the color (~10 min). The bound enzyme activity was determined by reading the absorbance at 450 nm.

Competitive Direct ELISA (CD-ELISA). A CD-ELISA using FmB_3−HRP as antigen marker was used to assess the presence of specific FmB_4 antibodies in the rabbit sera, for antibody characterization, and for analyzing *Fusarium* culture extracts. The protocol was essentially the same as previously described for fumonisin B_1 (Yu and Chu, 1996). The wells of microtiter plates were coated with 0.1 mL of either ammonium sulfate-precipitated anti-FmB_4 antiserum or purified antibody (10 μg/mL) at 4 °C overnight. After washing, the wells were blocked for nonspecific binding with 0.18 mL of 0.1% BSA in PBS for 30 min at 37 °C. The plates were washed again, and 0.05 mL of fumonisin standard solutions or diluted sample extracts was added to the wells, followed by the addition of 0.05 mL of FmB_3−HRP (diluted 1:500, 0.8 μg of protein/mL). The plates were incubated for 1 h at 37 °C and washed, and K-blue substrate (0.1 mL/well) was then added. The reaction was stopped by the addition of 0.1 mL of 1 M HCl after color development, and the absorbance at 450 nm was measured.

Culture Extraction and Analytical Recovery Test of *Fusarium* Culture Material. Various *F. moniliforme* strains grown on autoclaved cracked corn were supplied by Dr. J. F. Leslie, Department of Plant Pathology, Kansas State University, Manhattan, KS. A procedure similar to that of Yu and Chu (1998) was used for the extraction of fumonisins from the culture. In general, 2 g of dry corn culture materials was shaken overnight at room temperature with 20 mL of 50% (v/v) acetonitrile in water. An aliquot of the extract was then centrifuged at 10000*g* for 10 min, and the supernatant was diluted appropriately with PBS.

To assess the efficiency of the FmB_4−PAb-based CD-ELISA for the determination of fumonisins in *Fusarium* corn culture material, an *F. moniliforme* corn culture, which was found to contain very low levels of fumonisins in a FmB_1−PAb-based CD-ELISA (Yu and Chu, 1996), was used in the recovery study. In this study, 0.5 g of dry culture material was spiked with FmB_1 at levels of 0, 10, and 100 μg/g (in 0.5 mL of PBS). Duplicate samples were made at each toxin level. The spiked samples were left to stand in the cold room overnight and then extracted with 5 mL of 50% (v/v) acetonitrile in water as described above.

Analytical Recovery Test with Clean Corn as Matrix. Analytical recovery studies were also performed in clean corn to determine the lower detection limit of the FmB_4 −PAb-based CD-ELISA. An FmB_3/FmB_4-containing extract obtained from the *F. moniliforme* strain A0819, which has been shown to produce high amounts of FmB_3 and FmB_4 (Plattner et al., 1996), was used as spike material. The concentration of fumonisins in the spike solution was determined in five replicates with the FmB_4−PAb-based CD-ELISA before the corn sample was spiked. Typically, 2 g of fumonisin-negative dry corn meal sample used in a previous study (Yu and Chu, 1996) was spiked with appropriate amounts of the fumonisin-containing solution dissolved in 0.5 mL of PBS to give final concentrations of 0, 100, 500, and 1000 ppb. The spiked samples were left to stand at 4 °C overnight and were then extracted with 20 mL of 50% (v/v) acetonitrile in water as described above. The samples were analyzed in triplicate in the CD-ELISA.

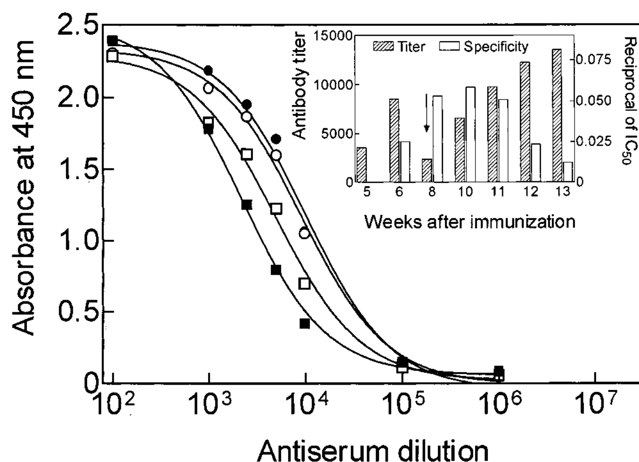

Figure 1. Representative antibody titration curves as determined with an FmB_3−OVA-based indirect ELISA. The antisera were obtained from the rabbit 5 (■), 10 (□), 12 (○), and 13 (●) weeks after initial immunization with FmB_4−KLH. The antibody titer is defined as the reciprocal of the antiserum dilution that gave an absorbance of 1.0 when analyzed in an indirect ELISA. (Inset) Antibody titers (open bars), defined as the reciprocal of the antiserum dilution that gave an absorbance of 1, and antibody avidity for FmB_4 (hatched bars), as determined in CD-ELISA and indicated as the reciprocal of IC_{50}. The black arrow indicates when a booster injection was made.

HPLC-ELISA Analysis of Culture Extracts. For the analysis of individual fumonisins, CD-ELISA was used as a postcolumn monitoring system under the conditions that we have previously described (Yu and Chu, 1998). The mobile phases consisted of solvent A [methanol/water/trifluoroacetic acid (TFA), 20:80:0.025, v/v] and solvent B (methanol/water/TFA, 90:10:0.025, v/v). After equilibration of the column with 100% A, 10−20 μL of filtered (0.45 μm) and diluted culture extract sample or fumonisin standard was injected. A gradient program running from 100% A to 100% B was applied over a period of 20 min. This was followed by a holding period of 10 min, and then gradually the program returned to 100% A in 20 min. The flow rate was 1 mL/min. Starting from the time of injection, 60−70 fractions of each 0.5 mL were collected and air-dried before they were redissolved in 0.5 mL of PBS and analyzed by CD-ELISA. Because a CD-ELISA format was used as the peak detecting unit, the obtained chromatograms are expressed as absorbance at 450 nm and, thus, consist of inverse peaks. The individual peaks were identified by comparison of the retention time with retention times of fumonisin standards run under identical conditions.

RESULTS

Production of Polyclonal Antibodies against FmB_4. Polyclonal antibodies against FmB_4 were generated in a rabbit immunized with FmB_4−KLH. The antibody titers of selected bleedings, as determined with an indirect ELISA using FmB_3−OVA as coating antigens, are shown in Figure 1. The rabbit exhibited an immunoresponse as early as 5 weeks after initial immunization and peaked at 13 weeks after immunization. The antibody titers and avidity of the antibodies for FmB_4, which is expressed as the reciprocal of the IC_{50} determined in CD-ELISA, for selected bleedings

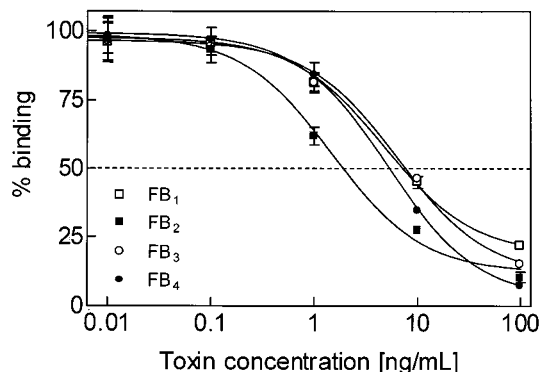

Figure 2. Cross-reactivities of the purified anti-FmB_4 antibody with FmB_1, FmB_2, FmB_3, and FmB_4 in a CD-ELISA using FmB_3−HRP as marker. Microtiter plate wells were each coated with 100 μL (10 μg/mL) of ion exchange chromatography-purified antibody collected 12 weeks after initial immunization. FmB_3−HRP (50 μL/well, 1:500) was used as enzyme marker to compete with 50 μL of fumonisin standard solution. The 50% inhibition concentrations (IC_{50}) of FmB_1, FmB_2, FmB_3, and FmB_4 were 7.1, 1.9, 7.6, and 5.3 ng/mL, respectively. All data were obtained from the average of triplicate independent experiments, and standard deviations were within the size of the symbols.

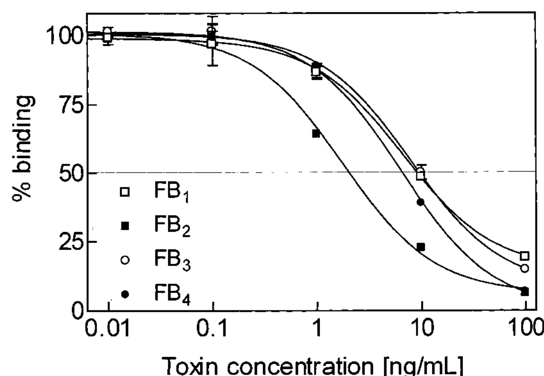

Figure 3. Cross-reactivities of the purified anti-FmB_4 antibody with FmB_1, FmB_2, FmB_3, and FmB_4 in a CD-ELISA using FmB_1−HRP as the competing enzyme marker. Microtiter plate wells were each coated with 100 μL of antibody (10 μg/mL). Standard solution (50 μL/well) and FmB_1−HRP (50 μL/well, 1:4000) was used in this assay. The IC_{50} values for FmB_1, FmB_2, FmB_3, and FmB_4 were 9.1, 2.0, 9.5, and 6.6 ng/mL, respectively.

over a period of 13 weeks are shown in the inset of Figure 1. It is apparent that the antibody avidity for FmB_4 peaked before the antibody titer reached the peak. The antibody titers were also determined with an FmB_1−OVA-based indirect ELISA, and the results were almost identical to those obtained with the FmB_3−OVA-based ELISA (data omitted). Thus, both FmB_3−OVA and FmB_1−OVA appear to have equal capabilities for the determination of antibody titer.

Characterization of the Purified Antibodies. For characterization of the antibody against FmB_4, the DEAE-cellulose-purified antiserum was used. Competition of four major fumonisins, that is, FmB_1, FmB_2, FmB_3, and FmB_4, for the binding of two types of enzyme markers, namely, FmB_1−HRP and FmB_3−HRP, with the purified antibody were examined in the CD-ELISA. Figure 2 shows that the concentration causing 50% inhibition of binding (IC_{50}) of the FmB_3−HRP to the solid-phase antibodies by FmB_4, FmB_1, FmB_2, and FmB_3 were 5.3, 7.1, 1.9, and 7.6 ng/mL, respectively, which corresponds to relative cross-reactivities of 100, 74, 280, and 70%, respectively. Using purified FmB_4

Table 1. Analytical Recovery of Fumonisin B_1 Added to *F. moniliforme* Culture Material by CD-ELISA

FmB_1 added	amount determined by CD-ELISA[a]							
	FmB_4−PAb-based				FmB_1−PAb-based			
(μg/g)	μg/g[b]	%	SD	CV%	μg/g[c]	%	SD	CV%
0	1.12		0.31	27.8	0.91		0.58	64.7
10	7.80	78.0	0.28	3.6	9.78	97.8	1.1	10.7
100	51.0	51.0	3.4	6.6	89.9	89.9	11.2	12.5
overall		64.5	14.4	22.4		93.6	10.9	11.76

[a] Mean and standard deviation of two samples, each run in at least duplicate measurements of two dilutions. [b] Because the recovery test is based on spiking with FmB_1, the cross-reactivity factor of 1/0.76 for FmB_1 is used to adjust the results obtained by using FmB_4 as standard in the FmB_4−PAb-based ELISA. The amounts shown in this column for the spiked samples were corrected for the blanks. For example, the amounts found in sample spiked with 10 and 100 ppm were 8.9 and 52.1 μg/g, respectively. [c] Using FmB_1 as standard in the ELISA; values for the spiked samples were corrected for the blanks.

Table 2. Analytical Recovery of Fumonisins Added to Clean Corn by the FmB_4−PAb-Based CD-ELISA[a]

fumonisin added[b] (ppb)	fumonisin recovered[a]				
	ppb[c]	%	SD (ppb)	CV%	95% CI
0	111.4		75.0	67.3	32.8−190.1 ppb
1000	918.4	91.8	69.3	7.5	84.6−99.1%
500	492.8	98.6	31.4	6.4	92.0−105.1%
100	106.1	106.1	4.4	4.1	101.5−110.7%
overall[d]		95.2	7.2%	7.6	90.6−99.8%

[a] The samples were run in triplicate assays; FmB4 was used as the standard. [b] The spike material consisted of a FmB_3/FmB_4-containing extract from the *F.* strain A0819 (Plattner et al., 1996). The concentration of fumonisins in the extract was determined in the FmB_4−PAb-based CD-ELISA. [c] The average amount found in the nonspiked sample (0 ppb) was subtracted from the amounts found in the spiked samples. [d] Data for the sample spiked at a 100 ppb level of fumonisin were excluded from the calculation of the overall results.

antibodies, the inhibition curves obtained in the FmB1−HRP system are shown in Figure 3; the IC_{50} values for FmB_1, FmB_2, FmB_3, and FmB_4 in this system were 9.0, 2.1, 9.0, and 6.5 ng/mL with relative cross-reactivities of 59, 310, 59, and 100%, respectively. These data are close to the values obtained by using FmB_3−HRP as enzyme marker. The FmB_4 antibodies nearly have the same affinity for FmB_1 as for FmB_3.

Analytical Recovery of *Fusarium* Culture Material Spiked with FmB_1. An analytical recovery study with the CD-ELISA was conducted using *Fusarium* culture material spiked with 10 and 100 μg of FmB_1/g of dry material. To compare the efficacy of the FmB_4−PAb-based CD-ELISA with that of the well-established FmB_1−PAb-based ELISA (Yu and Chu, 1996), the spiked culture samples were also analyzed with this assay. Results of the recovery studies are represented in Table 1. The overall analytical recovery for the FmB_4−PAb-based CD-ELISA developed in the present study was 65%, whereas the recovery using the FmB_1−PAb-based CD-ELISA was 94%.

Analytical Recovery of Clean Corn Spiked with FmB_3/FmB_4-Containing Extract. Results of the analytical recovery study on clean corn samples spiked with a FmB_3/FmB_4-containing extract are presented in Table 2. Even though the corn matrix was negative for fumonisins in the FmB_1−PAb-based CD-ELISA, some interfering background was found when the FmB_4−PAb-based CD-ELISA was used. This background level was subtracted from the results of the spiked samples.

Table 3. Determination of Total Fumonisins in Selected *F. moniliforme* Strains Analyzed with FmB_4−PAb-Based CD-ELISA and FmB_1−PAb-Based CD-ELISA

	fumonisin found (μg/g)	
strain used	FmB_4 ELISA	FmB_1 ELISA
6363	378	>1000
6364	106	208
6367	9	15
6373	370	687
6376	1.1	7
6461	432	>1000
6462	167	215
6463	198	228
6465	515	443
6468	115	85
A-0819	>1000	635

The overall recovery obtained in the range 100−1000 ppb was 99% (95% confidence interval = 95−103%). However, at the 100 ppb level, the recovery was >100% (106%). These results indicate that the lower detection limit should be between 100 and 500 ppb.

Analyses of *Fusarium* Cultures in CD-ELISA. Because the antibodies used in the FmB_1−PAb-based CD-ELISA do not cross-react with FmB_4, it is not possible to determine FmB_4 in *Fusarium* cultures by this assay. However, results obtained from studies showed that the FmB_4 PAb-based CD-ELISA developed in the present study could be used for screening strains that are found to contain relatively low levels of fumonisin (<1000 μg/g). Results of the analysis of relevant strains are given in Table 3. Although no new FmB_4-producing strains were found among the strains analyzed in this preliminary study, strain A-0819 was confirmed as a good FmB_4 and FmB_3 producer. This strain gave a considerably higher response in the FmB_4−PAb-based ELISA (>1000 ppm) than in the FmB_1−PAb-based ELISA (635 ppm). This is a good example of the strategy that could be used in future studies in screening cultures.

HPLC-ELISA Analyses of *Fusarium* Culture. Extract from the *F. moniliforme* strain A0819 was also analyzed in the HPLC-ELISA system to verify the presence of the FmB_4 as indicated above. Fractions obtained from the HPLC were analyzed with both the FmB_4−PAb-based and the FmB_1−PAb-based CD-ELISAs. Hence, two chromatograms were obtained. The lower part of Figure 4 represents these two chromatograms; the top part shows the chromatograms of FmB_3 and FmB_4 standards. By comparing the retention time of the peaks in the chromatogram of A0819 (23.0 and 25.5 min, as illustrated by a dotted line) with the retention times of FmB_3 standard (23 min) and FmB_4 standard (26 min), the peaks are clearly identified as FmB_3 and FmB_4, respectively. The absence of an FmB_4 peak in the chromatogram obtained in the FmB_1−PAb-based ELISA further proved that the data obtained from the FmB_4−PAb-based ELISA are due to the presence of FmB_4. Using peak areas of the FmB_3 and FmB_4 standards (20 ng/injection) in the top of this figure, this sample contains approximately 20.4 and 26.3 ng of FmB_3 and FmB_4 in 20 μL of the injected extract. The distributions of these two fumonisins in this sample were almost equal.

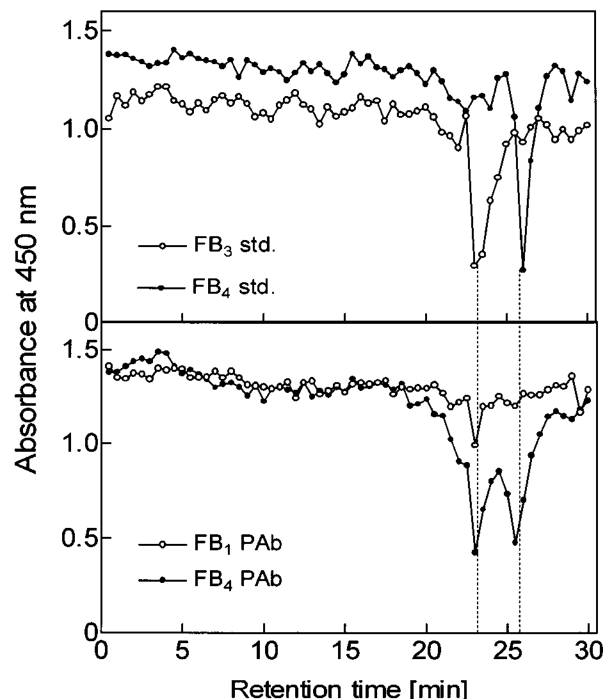

Figure 4. HPLC-ELISA chromatograms. The top panel shows the chromatogram obtained by analyzing standard solutions of FmB_3 and FmB_4 (20 ng each) in an FmB_4−PAb-based CD-ELISA. The retention times for FmB_3 and FmB_4 were 23 and 26 min, respectively, which clearly identify the peaks obtained by analyzing *Fusarium* strain A-0819 in the FmB_4−PAb-based ELISA (bottom figure and dashed lines). The chromatograms obtained by the FmB_1−PAb-based CD-ELISA lack the FmB_4 peak, which further confirms the presence of FmB_4 in the sample. The chromatograms were obtained by analyzing each of the 0.5 mL fractions collected from the HPLC (flow rate = 1.0 mL/min) with ELISA and plotting the measured absorbance against time (time for injection = 0).

DISCUSSION

Of all the antibodies generated against FmB_1, most of them are highly cross-reactive with other homologues such as FmB_2 and FmB_3 (Abouzied et al., 1995; Azcona-Olivera et al., 1992a,b; Elissalde et al., 1995; Fukuda et al., 1994; Iijima et al., 1996; Maragos and Miklasz, 1995; Newkirk et al., 1998; Usleber et al., 1994; Yeung et al., 1996; Yu and Chu, 1996, 1998, 1999a,b). No antibodies to date have been shown to cross-react with FmB_4. Thus, an ELISA for the detection of FmB_4 is not available. With the intention of developing an FmB_4 sensitive CD-ELISA, polyclonal antibodies against FmB_4 were produced and characterized in the present study. Using the same approach as for FmB1 (Yu and Chu, 1996), we conjugated FmB4 to KLH via the WSC method. Our result clearly demonstrates that the newly synthesized conjugate was an effective immunogen for generating antibodies against fumonisin. The antibodies with avidity toward FmB_4 were detected in the rabbit antiserum 6 weeks after initial immunization. Whereas the antibody titer peaked at the 13th week, antibodies with highest avidity for FmB_4 were found in the10th week's antiserum. This may be due to the presence of low-affinity FmB_4 antibodies that are able to recognize the solid-phase antigen carrier protein, the coupling region, or a conjugation byproduct.

The limited availability of FmB_4 made it necessary to use another fumonisin homologue as antigen marker. FmB_4 lacks hydroxyl groups at the C5 and C10 position that are present in the FmB_1 molecule. From a structural point of view, FmB_2 and FmB_3 have hydroxyl groups at the C-5 and C-10 positions, respectively, and are the homologues most closely related to FmB_4. Using

FmB_3−HRP as antigen marker in a CD-ELISA, the IC_{50} values for FmB_1, FmB_2, FmB_3, and FmB_4 were 7.1, 1.9, 7.6, and 5.3 ng/mL, respectively. These data show that the antibody cross-reacts with all four fumonisin homologues in the B series with the highest avidity for FmB_2 and lowest for FmB_1 and FmB_3. The present observations are consistent with the results obtained from a number of other studies that most antibodies raised against fumonisins with different conjugates have high avidity to FmB_2 (Chu, 1996b). Elissalde et al. (1995) proposed that the presence of the hydroxyl group at the C-5 postion facilitates antigen−antibody interaction. Our data on the antibodies raised against a conjugate containing FmB_4, which lacks the C-5 and C-10 hydroxyl groups, suggest that the backbone of the B series fumonisin plays a significant role in generating antibodies with specificity for this group of mycotoxins.

When FmB_1−HRP was tested as antigen marker, the obtained IC_{50} values were almost the same as those obtained using the FmB_3−HRP. This was expected because the cross-reactivities of the antibody toward FmB_1 and FmB_3 are almost identical. Although FmB_3−HRP was used throughout the present study, the FmB_1−HRP, in a future perspective, could be a suitable substitute. However, it should be pointed out that because FmB_4 was *not* used as marker in the assay described here, the true avidities of different fumonisins to the FmB_4 polyclonal antibodies generated in the present study need to be determined. The sensitivity of the assay will be improved if FmB_4−HRP is used as enzyme marker instead of FmB_3−HRP.

The analytical recovery of FmB_1 (10 and 100 μg/g) added to *Fusarium* culture material in the CD-ELISA was somewhat lower than the recovery obtained when the same samples were analyzed by the FmB_1−PAb-based CD-ELISA of Yu and Chu (1996) (65 and 94%, respectively). This might be partly due to inefficiency of the heterogeneous system in the FmB_4−PAb-based ELISA in which FmB_3 was used as marker and FmB_4 as standard to analyze for FmB_1. However, results from recovery studies in the FmB_4−PAb-based CD-ELISA on clean corn spiked with a FmB_3/FmB_4-containing extract showed an overall recovery of 99% (95% confidence interval = 95−103%). The recovery obtained at a toxin level of 100 ppb is questionable as it was >100% (106%). However, because the coefficients of variation were ∼5%, even at 100 ppb, the recovery was not alarmingly high. Thus, the detection limit with corn as matrix should be in the 100−500 ppb range. Because standard FmB_4 was not available in the required amounts, the concentration of a solution of extract containing FmB_3 and FmB_4 was determined in the FmB_4−PAb-based CD-ELISA and used as spike material. Consequently, the value for the overall recovery obtained in this recovery study does not provide information about accuracy as the concentration of the spike material was not determined by an independent accurate assay.

The intended use of the FmB_4−PAb-based CD-ELISA was to screen extracts of culture material of relevant *Fusarium* strains and to identify and characterize interesting fumonisin biosynthetic mutants. Desjardins et al. (1996) proposed that FmB_4 can be converted to either FmB_2 or FmB_3 by hydroxylation of C-5 or C-10, respectively, which again can be further converted to FmB_1. Therefore, mutant strains that fail to hydroxylate either at the C-5 or C-10 position from FmB_2 or FmB_3 or at both positions should accumulate FmB_4. When fumonisin is analyzed with an FmB_1−PAb-based ELISA, such mutant strains should contain lower levels (if any) of fumonisin than when analyzed in the FmB_4−PAb-based CD-ELISA developed in this study. On the basis of this strategy, the FmB_4−PAb-based CD-ELISA was evaluated as a screening assay for strains that are found to contain relatively low levels of fumonisin (<1000 μg/g) in the FmB_1−PAb-based ELISA. The levels of fumonisins found by analyzing extracts from a limited number of *Fusarium* strains in the FmB_4−PAb-based ELISA were both above and, mostly, below the levels determined in the FmB_1−PAb-based ELISA. Because of the different cross-reactivities toward FmB_{1-3} of the two antibodies [IC_{50} values of the FmB_1−PAb of Yu and Chu (1996) for FmB_{1-3} are 0.45, 0.72, and 25 ng/mL, respectively] and also the assay efficiencies and standards being used, there is no direct correlation between the results obtained by the two ELISAs, not even for samples solely containing fumonisins in which both antibodies are sensitive. Nevertheless, distinct differences in the fumonisin levels found in *F. moniliforme* strain A-0819 with these two assay systems could be due to the presence of FmB_4. It has has been reported that this culture produced both FmB_4 and FmB_3. The fumonisin level produced by this strain was >1000 μg/g in the FmB_4−PAb-based ELISA but was 635 μg/g with the FmB_1−PAb-based ELISA. The presence of FmB_3 in the culture extract of this strain was further verified with HPLC-ELISA (Yu and Chu, 1998). As a demonstration of the differences between the FmB_1− and FmB_4−PAb-based ELISAs, the HPLC fractions of the *Fusarium* strain A-0819 were also analyzed by the FmB_1−PAb-based ELISA. Although the chromatogram obtained in the FmB_4−PAb-based ELISA has two clear inverse peaks, the chromatogram obtained in the FmB_1−PAb-based ELISA showed only one small inverse peak as FmB_3. The absence of an FmB_4 peak in the FmB_1−PAb-based chromatogram is, in addition to the retention time for the FmB_4 peak in the FmB_4−PAb-based chromatogram, a further confirmation of the presence of FmB_4 in the analyzed sample. Using the HPLC-ELISA approach, we found that the ratio of FmB_3 and FmB_4 in extract of this culture was ∼0.78 (20.4 and 26.3 ng). Using the same strain supplied by Dr. John Leslie, Poling and Plattner (1996) isolated 277 and 66 mg of FmB_3 and FmB_4, respectively, from the corn culture extracts. However, because FmB_3 was used as the response factor in the analysis, they concluded that they may have underestimated the actual FmB_4 level.

In conclusion, the production and characterization of polyclonal antibodies against FmB_4 by use of an FmB_4−KLH conjugate to immunize a rabbit are reported herein. The antibodies were successfully applied in a CD-ELISA using FmB_3−HRP as enzyme marker. Because the antibodies highly cross-react with FmB_{1-4}, they could be a useful supplement to the conventional FmB_1 antibody-based ELISA, which is not sensitive to FmB_4. The newly developed FmB_4−PAb-based CD-ELISA could be used for extensive screenings of *Fusarium* strains with the purpose of identifying variants with unusual fumonisin production phenotypes. Other combinations of ELISAs sensitive to different fumonisin homologues might, likewise, be a potential way of identifying interesting fumonisin biosynthetic mutants.

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