Fiber-Optic Immunosensor for the Detection of Fumonisin B_1

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A fiber-optic immunosensor was developed to measure the mycotoxin fumonisin B_1 (FB_1). Monoclonal antibodies produced against FB_1 were covalently bound through a heterobifunctional silane to an etched 800 μm core optical fiber. An evanescent wave effect was utilized to excite fluorescein isothiocyanate labeled FB_1 (FB_1-FITC) molecules near the surface of the fiber. A direct competitive assay was used to measure FB_1 concentrations with the following steps: saturation of antibody binding sites by FB_1-FITC, competition of FB_1 and FB_1-FITC with displacement of the labeled toxin, and resaturation of binding sites by FB_1-FITC. The signal generated in the assay was found to be inversely proportional to the FB_1 concentration. This sensor exhibited a working range of 10−1000 ng of FB_1/mL, an IC_{50} of 70 ng/mL, and a limit of detection of 10 ng/mL. These values compared favorably with those for currently available ELISA techniques. The sensor exhibited cross-reactivity with fumonisin B_2 but did not react with hydrolyzed FB_1, sphinganine, or tricarballylic acid. Sensor performance was also found to be unaffected in a sample matrix of methanol/water-extracted corn.

Keywords: *Immunosensor; fiber optic; fluorescence; fumonisin; biosensor*

INTRODUCTION

Fiber-optic immunosensors have been used to detect a variety of compounds such as pesticides (Oroszlan *et al.*, 1993; Bier *et al.*, 1992; Anis *et al.*, 1993), bacterial toxins (Shriver-Lake *et al.*, 1993), environmental pollutants (Vo-Dinh *et al.*, 1992; Celebre *et al.*, 1992; Shriver-Lake *et al.*, 1995; Zhao *et al.*, 1995), and pharmaceuticals and serum analytes (Bluestein *et al.*, 1990; Devine *et al.*, 1995). They offer several advantages over other types of sensors: high specificity, freedom from electromagnetic interference, ease of miniaturization, real-time monitoring, biocompatibility, and adaptability for remote sensing.

One of the most promising types of fiber-optic sensors utilizes the evanescent wave effect. An evanescent wave is generated at the interface between an optical fiber and an outside lower refractive index material (e.g. cladding or liquid). When total internal reflection occurs in optical fibers, the light intensity cannot drop instantaneously to zero at the interface, but rather decays exponentially into the lower refractive index material. The penetration depth (defined as the distance from the interface where the intensity has dropped to 1/*e* of the initial value) depends upon the wavelength of light, the refractive indices of the two materials, and the angle of light incidence (Andrade *et al.*, 1985). A typical value for this depth is about 1000 Å (Rogers *et al.*, 1992). If the proper wavelength is selected, fluorescent molecules in this region can absorb energy from the evanescent wave and fluoresce. A portion of this fluorescence will be coupled back into the fiber and can be detected. By immobilizing antibodies to the surface of an optical fiber, an effective method of separating fluorescent antigens from the bulk solution can be achieved. Because only molecules in this region will be excited, fluorescent interference from the bulk solution is almost completely eliminated.

In this study, a fiber-optic evanescent wave immunosensor utilizing a direct competitive assay was developed for measuring the mycotoxin fumonisin B_1 (Figure 1). Unlike the repetitive hit-and-run fluoroimmunoassay for T-2 toxin (Warden *et al.*, 1987), this sensor uses an optical fiber with specific antibodies attached rather than agarose gel. Fumonisins, a potential health threat in both humans and animals, are primarily produced by *Fusarium moniliforme* and *Fusarium proliferatum*, common fungal contaminants of corn. The fumonisins have been shown to induce leukoencephalomalacia in horses (Wilson *et al.*, 1992; Kellerman *et al.*, 1990) and pulmonary edema in swine (Colvin and Harrison, 1992), have hepatotoxic and carcinogenic effects in rats (Gelderblom *et al.*, 1991), and may have a role in human esophageal cancer (Sydenham *et al.*, 1990). Currently available techniques to measure fumonisins involve extensive sample preparation (HPLC) or require multiple steps to complete (ELISAs). Fiber-optic sensors have shown promise as fast, simple methods that require little sample preparation.

MATERIALS AND METHODS

Materials. Plastic-clad silica fibers with an 800 μm core diameter were purchased from General Fiber Optics (Fairfield, NJ). Fluorescein 5-isothiocyanate (FITC) and 1-(trimethoxysilyl)-2-(*p,m*-chloromethyl)phenylethane were obtained from Molecular Probes Inc. (Eugene, OR) and United Chemical Technologies, Inc. (Bristol, PA), respectively. FITC-labeled goat anti-mouse IgG antibodies (GAM−FITC) were purchased from Jackson ImmunoResearch (West Grove, PA). Fumonisin B_1 (FB_1) was a gift from Ronald Plattner (USDA/ARS/NCAUR, Peoria, IL). All other chemicals were of reagent grade or better.

Monoclonal Antibody. Monoclonal anti-fumonisin B_1 antibodies were produced following methods previously described (Azcona-Olivera *et al.*, 1992; Chu *et al.*, 1995). One clone (reference no. P2A5-3-F3) was used for all experiments. Competitive direct ELISAs with this antibody were conducted as described previously (Maragos *et al.*, 1996), except with P2A5-3-F3 antibody coated at 200 ng/well and FB_1−horseradish peroxidase (FB_1-HRP) diluted 1:40 000. The concentration of free toxin required to displace 50% of the bound FB_1−HRP was 424 ng/mL for FB_1, 1109 ng/mL for FB_2, and 844 ng/mL for FB_3. This indicates that antibody from the P2A5-3-F3 clone cross-reacts with all three fumonisins.

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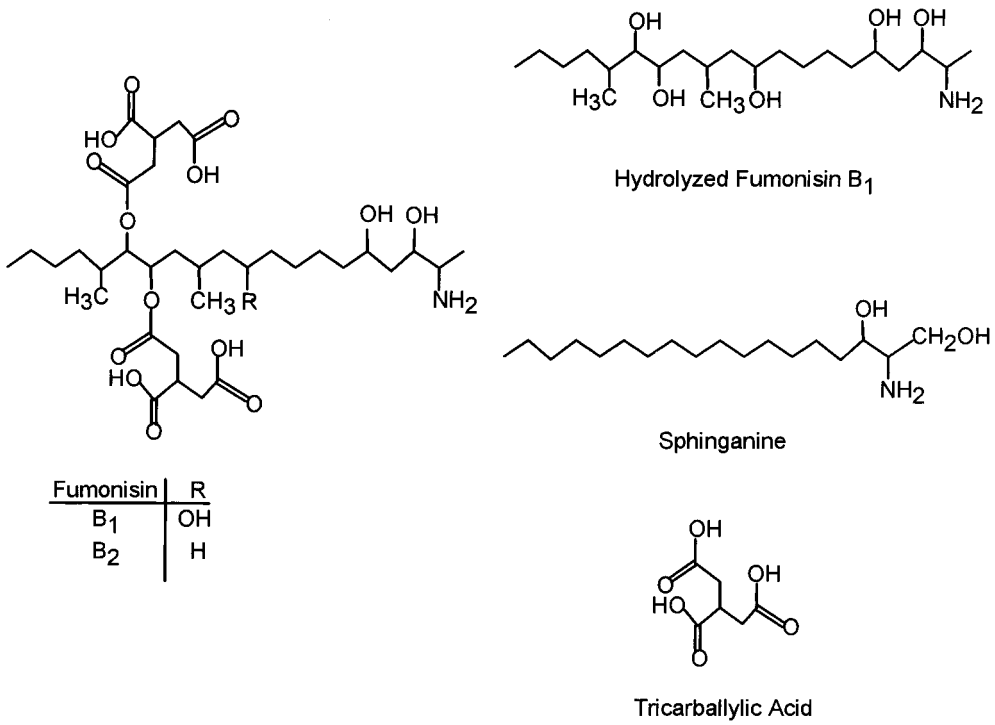

Figure 1. Molecular structures of fumonisin B_1, fumonisin B_2, hydrolyzed fumonisin B_1, sphinganine, and tricarballylic acid.

Optical Fiber Sensors. Fibers were cut into 17 cm lengths for each optic sensor. One end of the fiber was polished with a polishing fixture (Newport Corp., Irvine, CA) and aluminum oxide grit paper with 63, 9, 1, and 0.3 μm grit sizes. The sensor portion of the fiber had the cladding removed over a distance of 9 cm using a razor blade and was briefly dipped into concentrated sulfuric acid to remove residual cladding. To prevent loss of light from the fiber due to a V-number mismatch between the clad and unclad portions of the fiber (Anderson *et al.*, 1993), the unclad section was etched in hydrofluoric acid to a final diameter of 530 μm. A bifunctional silane, 1-(trimethoxysilyl)-2-(*p,m*-chloromethyl)phenylethane, was applied to the fiber as described previously (Pope *et al.*, 1993). Briefly, the fibers were coated with the silane (4% solution in 95% ethanol/water at pH 5.0) for 5 min, rinsed with absolute ethanol, and dried at 110 °C for 30 min. Anti-fumonisin B_1 antibodies were attached to the fiber through the silane layer by incubating the fiber in a 16 μg/mL solution of antibody in 10 mM phosphate-buffered saline (PBS) for 1 h (pH of the PBS was 7.4, and the temperature was 25 °C). The resulting sensors were rinsed with 10 mM PBS prior to use.

Antibody Coupling. Optical sensors prepared as described above were incubated with 3 μg of GAM−FITC/mL for 1 h at room temperature. The sensors were rinsed with 10 mM PBS following incubation to remove unbound GAM−FITC and incubated with 3 M potassium thiocyanate chaotropic agent for 1 h at room temperature. The fluorescence of the potassium thiocyanate solution was measured at 490 nm excitation and 520 nm emission on a fluorometer (SPEX Fluoromax, Edison, NJ).

Preparation of Labeled FB_1. Fumonisin B_1−fluorescein isothiocyanate (FB_1-FITC) conjugate was prepared as described previously (Maragos, 1995) with the following modifications: the dimethyl sulfoxide (DMSO) and borate buffer (BB), pH 9.5, ratio was reduced from 9:1 to 3:1, the BB concentration was increased from 50 to 100 mM, and the amount of FITC added was changed from 75 μL of 1.3 mM FITC in acetone to 100 μL of 10 mM FITC in DMSO. The FB_1−FITC conjugate was purified on an affinity column (1 mL bed volume) similar to those described previously (Ware *et al.*, 1994) but having a greater FB_1 capacity (20 μg). The column was washed with 10 mL of 10 mM PBS, the FB_1−FITC conjugate was diluted to 4 mL with 10 mM PBS and loaded onto the column, the column was rinsed with 10 mL of 10 mM PBS, and the conjugate was eluted with 4 mL of a 40:60 (v/v) mixture of methanol and 2 mM BB, pH 9.5, giving a final conjugate concentration of 4500 ng/mL as measured by HPLC (Maragos, 1995). Since a large excess of FITC was used in the conjugating reaction, it was assumed that essentially all of the FB_1 was conjugated and no attempt was made to remove residual free FB_1 from the conjugate.

Fluorometer. The experimental apparatus for the fluorometer was based on conventional optics similar to that described previously (Glass *et al.*, 1987). A schematic of the apparatus is shown in Figure 2a. Excitation light was provided by a 150 W xenon lamp, and the appropriate wavelength (490 nm) was selected by a monochromator. Light from the monochromator was focused onto an 800 μm diameter optical fiber (General Fiber Optics, Fairfield, NJ) with a collimator (General Fiber Optics). This fiber directed the excitation light to a plano-convex BK-7 glass lens ($f = 50$ mm; Melles-Griot, Irvine, CA) where it was collimated. Excitation light was turned 90° by a dichroic mirror (Omega Optical, Brattleboro, VT), which reflects at 490 nm, and the light was focused onto the fiber optic sensor with another plano-convex lens. Emission light (520 nm), generated by fluorescence of FB_1−FITC molecules bound to the anti-FB_1 antibodies, was partially coupled back into the sensor, collimated by the plano-convex lens, passed through the dichroic mirror, and focused onto another 800 μm optical fiber. This fiber directed the emission light through a 520 nm bandpass filter (Oriel Corp., Stratford, CT) onto another monochromator which selected at 520 nm. The signal generated by a photomultiplier was divided by the lamp reference signal to remove effects caused by variation of the lamp light intensity. The xenon lamp, monochromators, and photomultiplier were part of a Fluoromax fluorometer (SPEX Industries, Inc., Edison, NJ). The remaining instrumentation was built in-house.

Flow Cell. Samples were introduced to the fiber-optic sensor by means of a flow cell. A schematic of the flow cell is shown in Figure 2b (not to scale). The body of the flow cell consisted of a 50 μL glass capillary tube with low-pressure variable-bore tee connectors (Rainin Instrument Co., Inc., Ridgefield, NJ) at either end. Screw caps (not shown) on each arm of the tee connectors contained O-rings which formed a seal when the caps were tightened. The sensor was placed inside the capillary, and the appropriate caps of the tee connectors were tightened to seal the sensor in the capillary, forming a chamber. Tubing was connected to the remaining

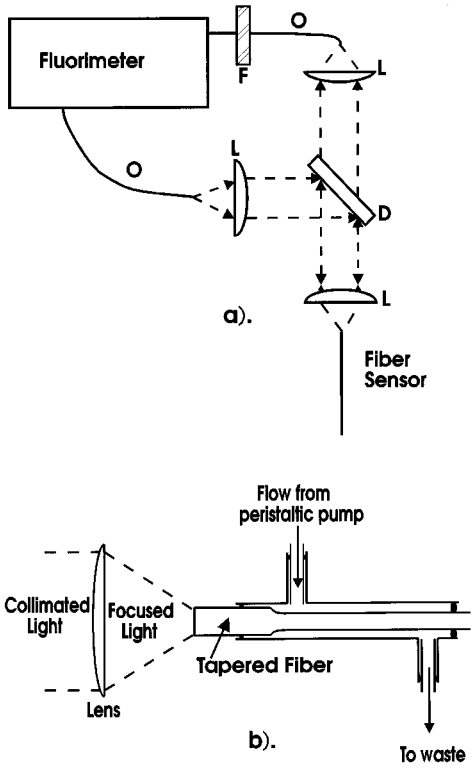

Figure 2. (a) Schematic diagram of optical apparatus for fiber-optic sensor: O, optical fiber (800 μm core diameter); L, BK-7 glass lens (f = 50 mm); D, dichroic mirror (reflectant at 490 nm, transparent at 520 nm); F, 520 nm filter (20 nm bandpass). (b) Schematic diagram of the fiber-optic flow cell.

ends of the tee connectors. A peristaltic pump (Cole-Parmer, Niles, IL) was used to pump samples through the chamber at a flow rate of 0.8 mL/min.

Assay. Fumonisin B_1−fluorescein isothiocyanate conjugate solution prepared as described above was diluted to 45 ng/mL with 10 mM PBS. For measurement of fumonisin standards, 100 μL stock solutions of purified FB_1 dissolved in acetonitrile/water (ratio 3:2 v/v) were mixed with FB_1−FITC solutions to obtain the appropriate FB_1 concentrations. In addition, a sample of FB_1−FITC containing 0 ng of FB_1/mL was also prepared by adding 100 μL of acetonitrile/water to account for possible acetonitrile effects on antibody binding. The assay for measurement of FB_1 was initiated by sealing a freshly prepared fiber into the flow cell and pumping a solution of 45 ng of FB_1−FITC/mL past the fiber until the signal reached steady state (approximately 20 min), indicating the antibody sites were saturated (Figure 3a). A sample containing a known concentration of FB_1 in FB_1−FITC solution was then pumped through the flow cell. Competition between FB_1 and FB_1−FITC in the solution for a limited number of binding sites resulted in a signal decrease proportional to the concentration of FB_1 (Figure 3b). The optical sensor was regenerated by pumping more FB_1−FITC through the flow cell to displace unlabeled FB_1 from the sensor until the original steady state value was again obtained (Figure 3c). After the initial sensor saturation, subsequent sensor regenerations took approximately 4 min. The procedure of alternating sample with FB_1−FITC solution was repeated for each sample. A single sensor was used for each set of FB_1 samples ranging from 0 to 100 000 ng of FB_1/mL.

Corn Extract Preparation. Corn retained on a no. 5 sieve (4 mm opening; Fisher Scientific Co., Pittsburgh, PA) was ground for 30 s in a Model M-2 Stein mill (Seedburo Engineering Co., Chicago, IL). A 25 g sample of ground corn was extracted with a mixture of 80% methanol and 20% water by shaking for 1 h as described previously (Bennett and Richard, 1994).

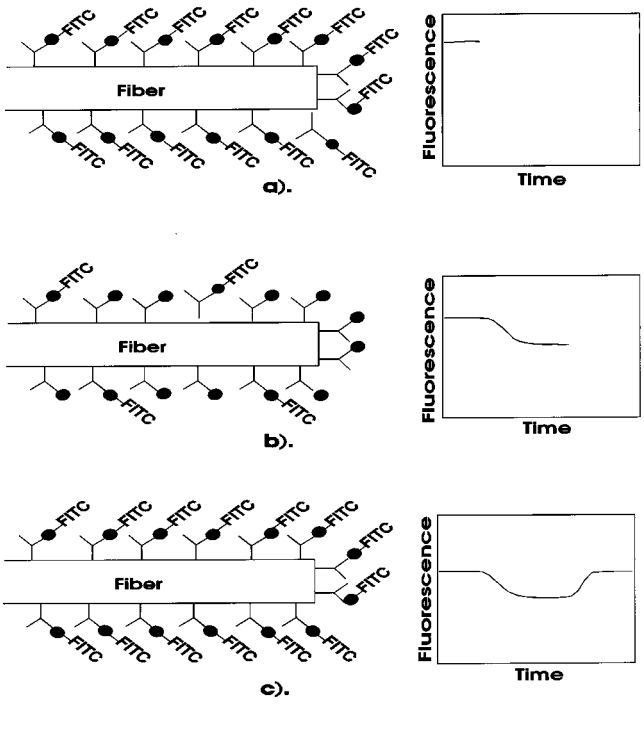

Figure 3. Direct competitive immunoassay: (a) saturation of antibody binding sites with FB_1−FITC; (b) competition of FB_1 and FB_1-FITC for antibody binding sites; (c) resaturation of antibody binding sites.

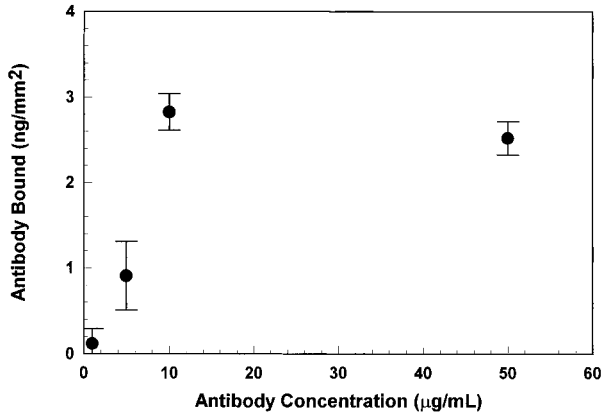

Figure 4. Effect of antibody concentration on the amount of antibody covalently bound to the sensor.

RESULTS AND DISCUSSION

Assay Properties. To facilitate development of an assay for FB_1, conditions for optimal sensor performance were examined, including the amount of antibody covalently attached to the fiber, the degree of nonspecific binding of FB_1−FITC to the sensor, the extent of photobleaching, and the reusability of the antibody-coated sensor. The amount of antibody covalently bound to the fiber was estimated by assuming that GAM−FITC bound univalently to the anti-FB_1 antibodies and that the potassium thiocyanate incubation removed most of the GAM-FITC (Tromberg *et al.*, 1987). Antibody binding to the fiber increased with increasing applied antibody concentrations up to 10 μg/mL and then leveled off (Figure 4). Optimum antibody binding of 2.5−2.8 ng/mm^2 occurred for applied antibody concentrations above 10 μg/mL and is comparable to values reported in the literature (Ogert *et al.*, 1992).

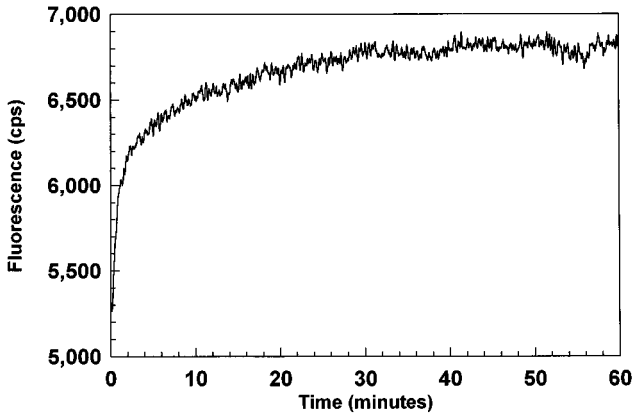

Figure 5. Time course signal from fiber-optic sensor while FB_1−FITC was pumped through the flow cell.

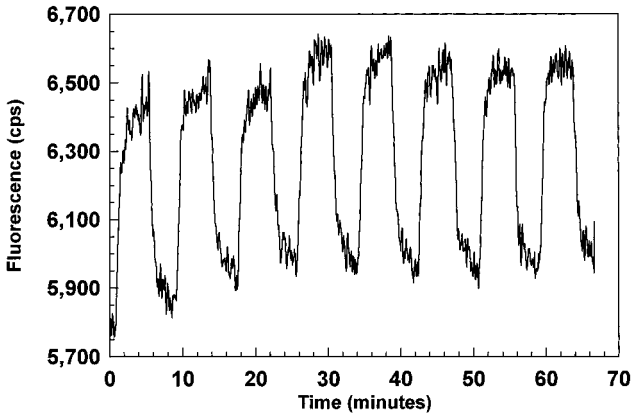

Figure 6. Fiber-optic sensor signal through eight displacement and regeneration cycles. Displacement was with 1000 ng of FB_1/mL.

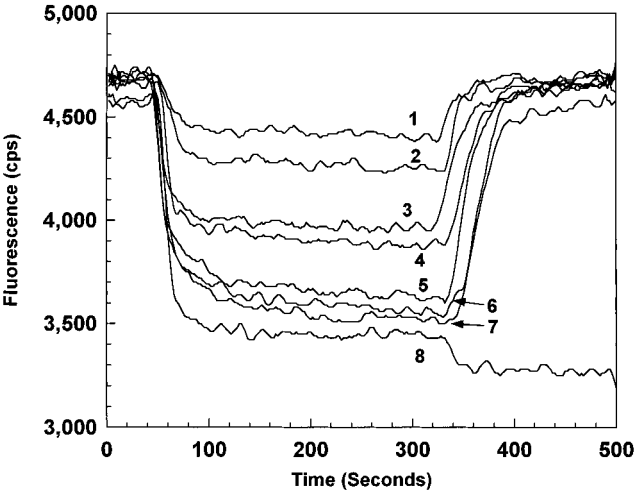

Figure 7. Fiber-optic sensor signal during the assay for FB_1 concentrations of (1) 0, (2) 10, (3) 50, (4) 100, (5) 500, (6) 1000, (7) 10 000, and (8) 100 000 ng/mL.

Nonspecific sensor binding was determined by coating a fiber with mouse IgG that was nonspecific for FB_1 and pumping FB_1−FITC past it in the flow cell. Signals generated by this sensor were typically 5% of the FB_1−FITC saturation values generated by sensors with a coating of specific antibody. Photobleaching caused by continuous exposure to excitation light was a concern because FITC is light sensitive. This was tested by pumping 45 ng of FB_1−FITC/mL through the flow cell for 1 h while continuously exposing the sensor to 490 nm light. There appeared to be no observable photobleaching, or if photobleaching was occurring, exchange of FB_1−FITC molecules was rapid enough to prevent it from being observed (Figure 5). Since there is one FITC molecule per FB_1 molecule, the increase in fluorescence can be directly related to binding of FB_1−FITC molecules to the antibody. From these data, the association kinetics of antigen−antibody binding can be determined (Glaser, 1993; Sadana and Sii, 1992). Measurement of antigen−antibody binding constants for free solution and immobilized antibodies could yield useful information about the effects of immobilization on antibody performance.

Most prior studies of fiber-optic sensors have used one fiber per assay. Because of fiber to fiber variability, this is not the most efficient method. To eliminate these effects, a single fiber could be used for multiple assays. A fiber sensor for imazethapyr herbicide was used for multiple assays (Anis *et al.*, 1993) by regenerating the sensor after contact with the antigen of interest; however, the signal at saturation decreased over time. This may have resulted because the antibody was not covalently attached to the fiber. Because covalent attachment was used in our study, an experiment was conducted to determine how stable the signal was to repeated regeneration cycles. The fluorescence signal (measured 250 s after introduction of FB_1−FITC) deviated less than 2% over eight regeneration and displacement cycles (Figure 6). Further experiments (data not shown) have revealed that the sensor could be regenerated up to 20 times before loss of sensitivity was seen.

The flow cell used in this study was designed to allow quick insertion and removal of sensors, to be reusable, and to be cost effective. Flow cell designs have been introduced having (a) flow-through cuvettes (Bier *et al.*, 1992; Anis *et al.*, 1993), which are reusable but fairly expensive, or (b) glass capillary tubes with the sensor glued in place (Ligler *et al.*, 1993), which are inexpensive but non-reusable. In addition, the second design requires that the glue harden before use and is not always guaranteed to be leakproof. The flow cell developed here provided leak-free operation while allowing the sensor to be inserted or removed from the cell in a matter of seconds. This design could also be reused indefinitely, and the materials to construct it were less expensive than a purchased flow-through cell.

Assay. A direct competitive assay format was utilized to measure concentrations of FB_1 standards (Figure 3). This assay consisted of three parts: (i) initial saturation of antibody binding sites with FB_1−FITC (approximately 20 min); (ii) addition of FB_1 with FB_1−FITC to allow competition for binding sites (4 min); and (iii) resaturation of binding sites with FB_1−FITC (4 min). The second two steps were repeated for subsequent samples. This assay is similar to previously described assays (Anis *et al.*, 1993; Zhao *et al.*, 1995) in which displacement of the bound antigen label with free antigen was more sensitive than inhibition of antigen label binding by free antigen. As expected with a competitive assay, the absolute fluorescence level was found to be inversely proportional to the FB_1 concentration (Figure 7). The decrease in signal observed for the 0 ng/mL sample resulted from residual (1%) acetonitrile present in all of the samples. This small amount of acetonitrile may have affected FB_1−FITC binding to the antibody or it may have caused quenching of the fluorescein fluorescence.

The reversibility of the assay was excellent for FB_1 concentrations of 1000 ng/mL and below as evidenced

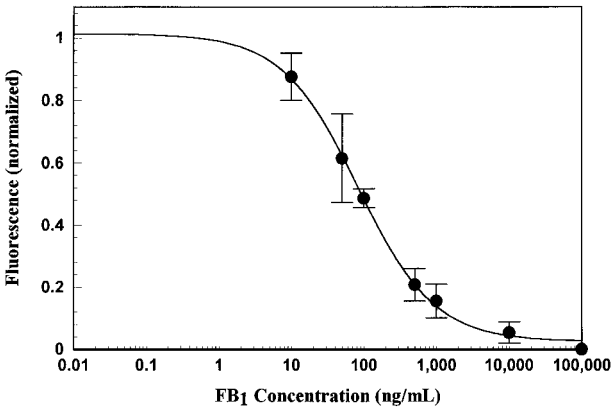

Figure 8. FB_1 displacement curve for the fiber-optic sensor. Data represent the average ± 1 standard deviation obtained from 10 separate fibers. A least-squares fit of the data to a logistic dose response is represented with a solid line.

by the return to the FB_1−FITC saturation level after exposure to the toxin (Figure 7). However, for FB_1 concentrations of 10 000 and 100 000 ng/mL, the sensor was not able to fully recover to the previous FB_1−FITC saturation values (sensor recovery for 100 000 ng/mL is not shown). Because of this, FB_1 concentrations of 10 000 and 100 000 were always introduced to the sensor last so that one sensor could be used for all of the samples. This indicates that the sensor can be used for multiple samples as long as the samples are diluted sufficiently to remain below 1000 ng/mL. Several types of washes were examined (basic conditions, acidic conditions, solvents such as methanol and acetonitrile, and combinations of solvents and pH conditions) to see if they could regenerate the sensor, but, in all cases, these treatments destroyed the ability of the antibodies to bind toxin and reduced the sensor signal to undetectable levels. Why the sensor performance was affected by high levels of toxin is unclear, but longer recovery times between samples may be the most viable method of regenerating the sensor after high level samples.

Fiber to fiber variability has been an ongoing problem in the development of fiber-optic sensors. In this study, a difference of 3000 cps in the maximum signal intensity has been observed between fibers (Figures 5−7). Variations in fiber diameter, antibody coating, and light launch angle and intensity, as well as internal and external fiber defects, all contribute to this variability (Wadkins *et al.*, 1995). To account for these effects, the data were normalized by assigning a value of 1 to the fluorescence value (the average fluorescence measured from 200 to 250 s after the sample is introduced) for 0 ng of FB_1/mL and a value of 0 for the 100 000 ng of FB_1/mL fluorescence value. It was assumed that there was no FB_1-FITC bound to the fiber in the presence of 100 000 ng of FB_1/mL. The binding curves produced from 10 different sensors were quite reproducible, and a logistic dose response described the data very well (Figure 8). The IC_{50} value (the concentration of toxin necessary to reduce the signal to half the toxin-free value) for this sensor was 70 ng/mL (97 nM). The working range of the sensor was from 10 to 1000 ng of FB_1/mL (13.9−1390 nM), and the limit of detection (signal to noise ratio of 3) was 10 ng/mL (13.9 nM). The working range and limit of detection are 1−2 orders of magnitude higher than those seen in the literature for some low molecular weight compounds, such as atrazine, terbutryn, and imazethapyr (Oroszlan *et al.*, 1993; Bier *et al.*, 1992; Anis *et al.*, 1993, respectively), but are comparable to the working ranges and detection limits obtained in other studies for low molecular weight compounds such as cocaine, trinitrotoluene, and polychlorinated biphenyls (Devine *et al.*, 1995; Shriver-Lake *et al.*, 1995; Zhao *et al.*, 1995, respectively). In addition, the sensitivity of this sensor compares favorably to literature values for measurement of FB_1 using an ELISA format with different anti-FB_1 antibodies (Azcona-Olivera *et al.*, 1992; Fukada *et al.*, 1994) having reported values of 50 and 10 ng/mL for limits of detection, respectively. To date, all monoclonal antibodies produced against FB_1 have had similar sensitivities (IC_{50} values of 65−790 ng/mL), although some highly sensitive polyclonal antibody based ELISAs (IC_{50} of 0.6 ng/mL) have been produced (Usleber *et al.*, 1994). Use of sensitive polyclonal antibodies or further improvement of available monoclonal antibodies should improve the sensor response.

Cross-Reactivity of the Sensor. To determine the specificity of this sensor, it was tested against several compounds (Figure 1) structurally similar to FB_1. The sensor had similar reactivity to fumonisin B_2 (IC_{50} of 221 ng/mL) as it did to FB_1 (IC_{50} of 70 ng/mL). This property will be valuable in the measurement of samples, for it will allow the combined fumonisin concentration to be estimated. The sensor did not react to sphinganine, tricarballylic acid, or hydrolyzed fumonisin B_1 even at 1000 ng/mL levels.

Sensor Response in Corn Extracts. To test the performance of the sensor in a real sample matrix, ground corn was extracted with an 80:20 methanol/water mixture and filtered to remove the corn. The resulting extract was diluted with 10 mM PBS containing 45 ng of FB_1−FITC/mL in either a 1:10 or a 1:20 ratio. No further treatment of the extracts was given. The 1:10 or 1:20 diluted corn extracts were spiked with FB_1 stock solutions to obtain concentrations ranging from 0 to 10 000 ng of FB_1/mL. The resulting samples were assayed as described above for the buffer samples. The IC_{50} values for the 1:10 and 1:20 dilution extracts were 520 and 124 ng of FB_1/mL, respectively. The limit of detection for the 1:10 dilution was approximately 25 ng of FB_1/mL, and the limit for the 1:20 dilution was 10 ng of FB_1/mL. These results indicate that while the matrix interferes with FB_1 measurement in the 1:10 dilution extract, sensor performance is virtually unimpeded with the 1:20 dilution extract. Application of the sensor to the analysis of corn is currently being investigated.

In conclusion, a fiber-optic sensor was successfully developed to measure fumonisin concentrations in buffered solution. This sensor has a working range of 10−1000 ng/mL, an IC_{50} value of 70 ng/mL, and a limit of detection of 10 ng of FB_1/mL. This detection limit compares favorably with the currently available techniques for measuring FB_1 concentrations, and the use of more sensitive anti-FB_1 antibodies will further improve the limits of detection. The fiber-optic sensor was highly selective toward fumonisins and did not cross-react with structurally related compounds that were tested. Performance of the sensor in the presence of a matrix of 1:20 diluted corn extract was similar to that with buffer. In addition, the flow cell developed in this study makes sample introduction simple and allows the sensor to be inserted and removed very quickly and easily. The ability to reuse the sensor eliminates problems with fiber to fiber variability commonly encountered in other fiber-optic sensor applications. While

this sensor is being developed for application as a screening tool for fumonisins in corn-based foods and feed, the techniques developed here can be readily adapted to analysis of other types of mycotoxins by immobilizing antibodies against other mycotoxins to the fiber.

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