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Development of Surface Plasmon Resonance-Based Immunoassay for Aflatoxin B_1

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Aflatoxins are a group of highly toxic fungal secondary metabolites that occur in *Aspergillus* species and may contaminate foodstuffs and feeds. Two different anti-aflatoxin B_1 antibodies were examined to develop a surface plasmon resonance (SPR)-based immunoassay to aflatoxin B_1. A conjugate consisting of aflatoxin B_1−bovine serum albumin (BSA) was immobilized on the dextran gel surface. Competition between immobilized aflatoxin B_1 conjugate and free aflatoxin B_1 in solution for binding to antibody injected over the surface formed the basis for the assay. Regeneration of the antibody from the immobilized conjugate surface is essential for the development of such an inhibitive immunoassay. Problems were encountered with the regeneration of the sensor surface, due to the high-affinity binding of the antibodies. Conventional regeneration solutions consisting of low concentrations of NaOH and HCl worked to a degree, but regeneration was at the expense of the integrity of the immobilized conjugate. A polyclonal anti-aflatoxin B_1 antibody was produced and was found to be regenerable using an organic solution consisting of 1 M ethanolamine with 20% (v/v) acetonitrile, pH 12.0. This combined high ionic strength and extreme pH, as well as chaotrophic properties and allowed the development of an inhibitive immunoassay. The assay had a linear range of 3.0−98.0 ng mL^{-1} with good reproducibility.

Keywords: *Aflatoxin B_1; surface plasmon resonance; regeneration; inhibitive immunoassay*

INTRODUCTION

Aflatoxins were discovered in 1960 following the deaths of several thousand turkey poults throughout England, due to consumption of contaminated Brazilian groundnut meal (Murray et al., 1982). They are a group of highly toxic fungal secondary metabolites that occur in *Aspergillus* species (O'Kennedy and Thornes, 1997). The fungus contaminates foodstuffs and feeds as well as crops such as maize, cottonseed, peanuts, and tree nuts during growth but particularly while in storage. Contamination is most common in tropical and subtropical countries where humidity is high, and, therefore, favorable conditions exist for the fungus to grow. Aflatoxins are members of the coumarin family, and the most significantly occurring compound is aflatoxin B_1 (AFB_1), which is produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Other aflatoxins, designated B_2, G_1, and G_2, are also produced, but AFB_1 is generally present in the largest quantity and is the

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most toxic. Aflatoxin M_1 (AFM_1) is a hydroxylated metabolite of AFB_1, which is excreted in the milk of dairy cattle after they consume contaminated food. AFB_1 is a very potent carcinogen and has been linked to human hepatocellular carcinoma. The international agency for research on cancer regards it as a human carcinogen (Ward et al., 1990). Links between dietary exposure to aflatoxins and increased risks of primary hepatocellular carcinoma have been reported in several ethnic groups (Gerbes and Caselmann, 1993; Harrison et al., 1993).

Due to the widespread occurrence of the toxin-producing fungi in cereals, great efforts have been made to develop rapid and sensitive methods for the detection of aflatoxins. Thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) have been the traditional methods used (Nawaz et al., 1995), but these can lack sensitivity and consistency due to the number of steps required in sample preparation and cleanup.

The applications of antibodies in aflatoxin analysis includes sample cleanup associated with chromatographic methods and their use in immunoassays (Langone and Van Vunakis, 1976; Chu and Ueno, 1977). Enzyme-linked immunosorbent assay (ELISA) is an analytical technique that exploits the sensitivity and specificity of the antibody−antigen interaction. Because aflatoxins have a low molecular mass (i.e., <1000 Da), they must be covalently linked to an immunogenic carrier molecule such as a protein [e.g., bovine serum albumin (BSA)], which will elicit a strong immune response following immunization. The protein conjugate is also used at the screening and analytical stages of antibody production. Monoclonal and polyclonal antibodies have been produced using an aflatoxin B_1−BSA (AFB_1−BSA) conjugate (Ward et al., 1990). Although immunoassays (and ELISA in particular) have found widespread use in aflatoxin analysis, the availability of rapid and simple biosensor procedures could significantly improve the efficiency of routine surveillance programs.

Biospecific interaction analysis (BIA), employing biosensors is able to measure biospecific interactions (e.g., antigen−antibody binding) in "real-time". A commercially available instrument (BIAcore) employs the principle of surface plasmon resonance (SPR) (Quinn and O'Kennedy, 1999). It continuously detects changes in the refractive index of an antibody and free toxin solution close to the surface of the sensor chip. Aflatoxin conjugate is covalently immobilized onto the surface of the sensor chip and antibody and free toxin are allowed to flow continuously over the surface. The conjugate and free toxin in solution compete for binding to the antibody in solution. As antibody binds to the conjugate, the refractive index of the buffer in contact with the sensor chip changes. The change in refractive index is measured by SPR. Continuous monitoring of the resonance angle gives a change in the refractive index of the buffer solution close to the metal film surface. This change is then detected and quantified [as response units (RU)] by the instrument as a sensorgram. Approximately 1000 RU is equivalent to a mass change in the surface concentration of 1 ng mm^{-2} (Stenberg et al., 1991). After the binding interaction occurs, the bound antibody can be removed using chaotrophic reagents, which allow the sensor surface to be used repeatedly. BIAcore has been used for applications such as kinetic analysis (Malmborg and Borrebaeck, 1995) and inhibition immunoassays (Wagner et al., 1995). It was also previously used in the detection of mycotoxins (Van der Gaag et al., 1999), where aflatoxin B_1 was directly immobilized onto the dextran gel surface for use in an inhibitive immunoassay. It was found that the assay was sensitive enough for the detection of aflatoxins in food and feed. SPR-based immunoassays have also been developed for other mycotoxins, such as fumonisin B_1 (Mullett et al., 1998).

Because aflatoxins have a low molecular mass, the mass change caused by binding to the sensor surface may be too small to result in a significant change in refractive index. As a result, in the development of a quantitative method for the detection of aflatoxins and other low molecular mass compounds, it is necessary to use an indirect sensing method. This can either be **competitive**—in which standard samples and a high molecular mass hapten−conjugate compete for binding sites on an immobilized antibody surface or **inhibitive**—in which sample is incubated together with antibody and the mixture is passed over an immobilized conjugate surface for binding of remaining free antibody. Effective regeneration of the sensor surface is necessary for both of these formats if they are to be used routinely. The antibody−antigen interaction can often be very strong and, therefore, very difficult to dissociate, despite the use of strong chaotrophic reagents. When an antibody is selected for use in a regenerable immunosensor, it is necessary to choose one with a moderate affinity to facilitate easy regeneration of the sensor surface. If the choice of antibody is limited, alternative immobilization strategies can be used (Quinn et al., 1999).

This paper describes the quantification of free aflatoxin, using an inhibitive indirect sensing method on BIAcore. It illustrates how ELISA-based formats may be used for the selection of antibodies for use in inhibitive sensor-based regenerable assay formats. As a result of the strong binding interactions of these anti-aflatoxin antibodies, regeneration of the sensor surface was problematic. Two different anti-aflatoxin antibodies were evaluated for use. One of the antibodies was not suitable for use in a regenerable format. However, a polyclonal antibody preparation and an assay with suitable regenerable characteristics were successfully developed.

MATERIALS AND METHODS

Caution: Aflatoxin B_1 is carcinogenic and should be handled with extreme care.

All reagents and chemicals were supplied by Sigma Chemical Co. (Poole, Dorset, U.K.), unless otherwise stated. Carboxymethylated dextran was obtained from Fluka Chemicals (Gillingham, Dorset, U.K.). BIAcore 1000 and CM5 sensor chip were both supplied by BIAcore AB (Uppsala, Sweden).

Source of Antibodies. Two different antibodies produced against aflatoxin B_1 were used in the analysis. The two polyclonal preparations were designated antibody 1 (Sigma Corp.) and antibody 2 [Dublin City University (DCU)].

Rabbit Immunizations and Antibody Preparations. An adult New Zealand white female rabbit was immunized with an emulsion (1 mL), consisting of 200 μg mL^{-1} of AFB_1−BSA (supplied by Sigma Chemical Co.) conjugate mixed 1:1 with Freund's complete adjuvant. Once an antibody titer of greater than 1/100000 was reached, the rabbit was exsanguinated, and the serum was collected, allowed to clot for 2 h at room temperature, and stored overnight at 4 °C. It was then centrifuged at 4000 rpm for 20 min, and the supernatant was collected and stored at −20 °C until required for use.

The antibodies were purified from the serum using a 45% (w/v) saturated ammonium sulfate precipitation, followed by protein G affinity chromatography. Subtractive immunoaffinity chromatography was carried out using a Sepharose column containing immobilized BSA. This removed all of the "BSA-binding" antibodies.

Competitive ELISA for Detection of Aflatoxin B_1. Microtiter plates (Nunc Immunoplate Maxisorp, Gibco Ltd., Uxbridge, U.K.) were coated by adding 100 μL of AFB_1−BSA conjugate dissolved in phosphate-buffered saline solution (PBS, pH 7.3, 0.15 M NaCl) to each well. The plates were incubated overnight at 4 °C. The plates were emptied and washed six times, three times with PBS−Tween [0.05% (v/v) Tween 20; PBST] and three with PBS only. The plate was then blocked by addition of 100 μL per well of PBS containing 2% (w/v) milk powder and incubated for 1 h at 37 °C. (For both coating and blocking of plates, incubation steps could also be carried out at 37 °C for 1 h or 4 °C overnight.)

The optimal dilution of the various antibodies and the optimal conjugate concentration for use in competitive ELISA were determined by testing doubling dilutions of antibody against decreasing concentrations of conjugate. This protocol was carried out in the absence of free toxin. From the titre curves obtained, the antibody dilution that gave half the maximum absorbance and the lowest conjugate concentration that provided sufficiently high absorbances were chosen.

Stock aflatoxin B_1 solution was prepared at a concentration of 2 mg mL^{-1} in methanol and diluted in PBS containing 5% (v/v) methanol to produce a set of standard solutions ranging in concentration from 10 pg mL^{-1} to 50 μg mL^{-1}.

The plates were washed as before, and 50 μL of anti-AFB_1 antibody was added into each well with 50 μL of aflatoxin B_1 standards. The plate was incubated for 1 h at 37 °C. After washing, 100 μL of horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody, diluted in PBS, was added to each well and incubated for 1 h at 37 °C. The plates were emptied and washed. One hundred microliters of substrate [0.4 mg mL^{-1} *o*-phenylenediamine (o-PD), in 0.05 M phosphate citrate buffer, pH 5.0, and 0.4 mg mL^{-1} of urea hydrogen peroxide] was added into each well and incubated for 30 min at 37 °C. All absorbance values were measured on a Titertek Twinreader Plus plate reader at 405 nm.

Measurement of Cross-Reactions. Antibody 2 was assayed with a range of standards of aflatoxins B_1, B_2, M_1, M_2, G_1, G_2, B2a, and G2a. Standard curves for each of the aflatoxins were produced as already described for the competitive ELISA for detection of AFB_1. The results were normalized and plotted. The slope of the linear range of the standard curve for each toxin was expressed as the percentage of the slope of the line for binding to AFB_1.

Use of ELISA To Examine the Efficiency and Effects of Regeneration on an Immobilized AFB_1—BSA Surface. In experiments to examine the efficiency of regenerating conjugate coated at a surface, 96-well microtiter plates were coated with 50 μg mL^{-1} AFB_1−BSA and blocked with 100 μL/well of PBS containing 2% (w/v) milk powder.

After washing, 100 μL/well of a 1/5000 dilution of antibody 2 (anti-AFB_1) in PBS was added to the plates and then incubated at 37° C for 1 h.

A set of solutions of 1 M ethanolamine containing various percentage concentrations of acetonitrile was then prepared. Antibody solution was aspirated from the plates, which were washed three times in PBS−Tween [0.05% (v/v) Tween 20] (PBST) and once in PBS. One hundred microliters of each of the different ethanolamine−acetonitrile solutions was then added to wells, and the plates were incubated for 10 min at room temperature. Following incubation, the plates were washed three times in PBST and once in PBS.

One hundred microliters per well of appropriate HRP-labeled antispecies antibody−1/5000 in PBS−was then added, and the plates were incubated at 37° C for 1 h. After three washings in PBST and one in PBS, substrate (as described above) was added, and the absorbance of wells at 405 nm determined.

To examine the effect of the regeneration reagent upon the conjugate surface, the assay was repeated as before, except that the ethanolamine−acetonitrile solutions were added to the wells immediately after the blocking step. After washing, antibody 2 was added, incubation was carried out for 1 h at 37 °C, and the plate was washed as before. HRP-labeled antispecies antibodies were then added, and the plates were washed and incubated as previously. Finally, substrate was added and the absorbance measured.

Coupling Reaction of AFB_1—BSA to CM-Dextran Gel. The carboxymethylated dextran (CM-dextran) matrix was activated by mixing equal volumes of 100 mM *N*-hydroxysuccinimide (NHS) and 400 mM *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) and injecting the mixture over the sensor chip surface for 7 min at a flow rate of 5 μL min^{-1}. The interactant to be immobilized (concentration of 50−200 μg mL^{-1}) was dissolved in 10 mM acetate buffer (at the required pH) and injected over the surface for 20 min at a flow rate of 2 μL min^{-1}. The unreacted sites on the sensor chip surface were then capped by injection of 1 M ethanolamine, pH 8.5, for 7 min.

Sample Preparation for Sensor Analysis. The antibodies were diluted in Hepes-buffered saline solution (HBS running buffer, pH 7.4) containing 100 μg mL^{-1} CM-dextran. The latter was used to remove nonspecific interactions between the antibody and the CM-dextran matrix on the sensor surface. These solutions were preincubated for 1 h at 37° C. All buffers and solutions used were made up using ultrapure water, degassed and sterile filtered.

Regeneration. Regeneration of the surface of the chip was carried out using 10−100 mM HCl/NaOH or 1 M ethanolamine with 20% (v/v) acteonitrile, pH 12.0.

AFB_1 Preparation for Use in Inhibitive Assay. A 2 mg mL^{-1} solution of free AFB_1 was prepared in methanol. Standards were prepared in PBS containing 5%(v/v) methanol, at concentrations ranging from 0.762 and 97.65 ng mL^{-1}. Each sample was incubated with an equal volume of a 1/50 dilution of anti-aflatoxin antibody for 10 min and then passed over the surface of the chip. This was carried out three times for each concentration.

RESULTS

Antibody Analysis Using Immobilized AFB_1—BSA Conjugate Surface. The binding of polyclonal antibody 1 to an AFB_1−BSA conjugate surface was examined. A 200 μg mL^{-1} solution of AFB_1−BSA in 10 mM sodium acetate, pH 3.9, was immobilized on the sensor surface. NaOH (100 mM) was chosen as the regeneration agent, as HCl is generally not an effective regeneration reagent without being accompanied with base treatment. Approximately 2000 RU of AFB_1−BSA was immobilized on the surface.

The removal of bound antibody 1 from this surface using increasing molarities of NaOH was investigated. Only 100 mM NaOH was found to give complete regeneration. Figure 1 shows repeated binding/regeneration cycles, using a 1/100 dilution of antibody and 100 mM NaOH with various contact times. Regeneration with a 10-min injection of 100 mM NaOH seemed to be the most effective (the flow rate here was reduced to 2 μL/min, to minimize sample consumption).

In an attempt to develop a quantitative method for estimation of free AFB_1, a fresh AFB_1−BSA conjugate surface was immobilized and a range of standards of free toxin (0−40 μg mL^{-1} in PBS) prepared. The standards were then mixed and incubated with an equal volume of antibody 1 (1/100 in PBS). After a 10-min incubation period, the mixture was passed over the chip. A 10-min injection of 100 mM NaOH was used for regeneration of the surface. The flow rate used in the assay was 2 μL min^{-1}. It was not possible to construct

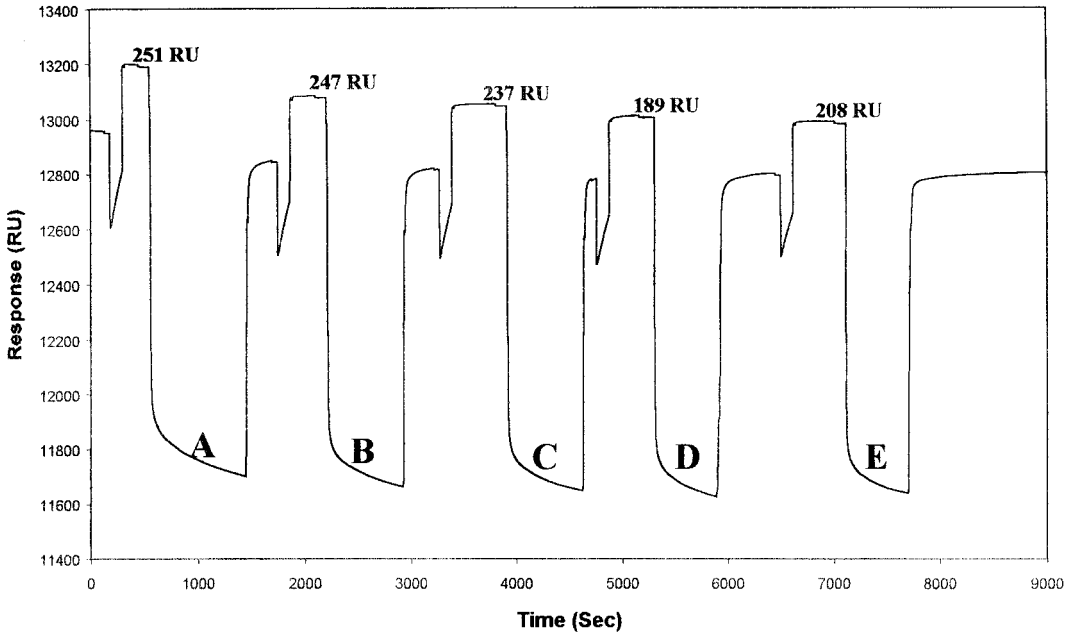

Figure 1. Sensorgram showing binding/regeneration cycles for antibody 1 bound to immobilized AFB_1−BSA. Antibody at a 1/100 dilution was injected over an AFB_1−BSA conjugate surface, and 100 mM NaOH was then passed over the chip. The steps indicate the contact times at which the regeneration reagent was injected over the bound antibody surface to optimize the conditions for the removal of antibody from the surface. They were as follows: (A) 15 min; (B, C) 12 min; (D, E) 10 min. The antibody-binding signal decreases after conditions A−C, but the response after condition D increases slightly, indicating that a contact time of 10 min is optimal for this concentration of base.

a standard curve using the results (data not shown) because of high variability, although the method worked in principle, with the change in response inversely proportional to the concentration of free toxin. Poor regeneration of the surface appears to have been responsible for the poor assay performance. The negative relative response values after regeneration with 100 mM NaOH show that this strategy, which was optimized for the removal of 200−300 RU of bound antibody, was damaging the immobilized surface when lesser amounts of IgG were bound.

Competition ELISA for AFB_1 Using Antibody 2. As it was not possible to develop an inhibitive SPR-based assay with the antibody analyzed, a polyclonal antibody to aflatoxin B_1 was produced using aflatoxin B_1−BSA conjugate as immunogen. After a titer level of 1/200000 was reached, a 45% (w/v) saturated ammonium sulfate precipitation was carried out on the serum. The anti-AFB_1 antibodies were then isolated using protein G affinity chromatography. To ensure that the isolated antibodies did not bind to the BSA part of the toxin conjugate, subtractive immunoaffinity chromatography was carried out. This entailed using a Sepharose column to which BSA was immobilized with removal of all of the antibodies binding to BSA. An inhibition ELISA was developed, with a conjugate coating concentration of 20 μg mL^{-1} and an antibody dilution of 1/10000. Standards of free aflatoxin were made up and were diluted in PBS containing 5% (v/v) methanol. They ranged in concentration from 0.762 to 50000 ng mL^{-1}. The intraday assay was carried out five times for each concentration on the same day. Figure 2 shows the relationship between absorbance (405 nm) and the concentration of free AFB_1 (ng mL^{-1}). The linear range for the assay was found to be between 12 and 25000 ng mL^{-1}, illustrating that the antibody could detect relatively low concentrations of free toxin. The correlation coefficient value (R^2) was found to be 0.99. Interday variation studies were also carried out by

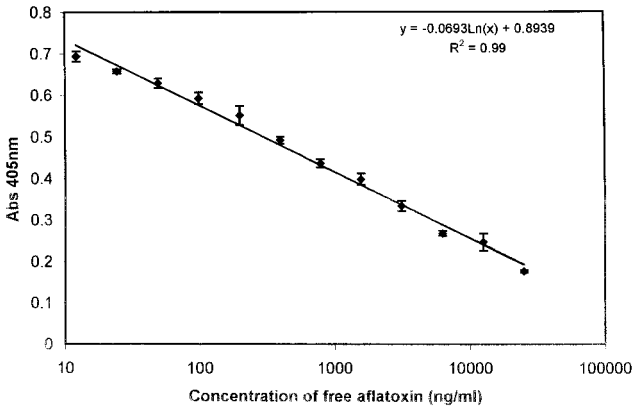

Figure 2. Competitive ELISA, with antibody 2, for determination of optimal range of detection of free AFB_1. The linear range of detection was 12.0−25000 ng mL^{-1}. Error bars on each calibration point indicate the standard deviation of the mean of five measurements.

Table 1. Cross-Reactions of Antibody 2 Observed to Seven Other Aflatoxins

aflatoxin	% cross-reactivity	aflatoxin	% cross-reactivity
B_1	100	M_1	35.09
B_2	67.5	M_2	1.67
G_1	65.5	B_{2a}	33.41
G_2	50.13	G_{2a}	15.53

assaying five sets of each standard on five different days. The assay was found to be reproducible within this range (12.0−25000 ng mL^{-1}) over the 5 days.

Specificity of Antibody 2. Antibody 2 did not react specifically with AFB_1. It had high cross-reactivities to AFB_2 (67.5%), AFG_1 (65.5%), and AFG_2 (50.13), whereas it cross-reacted at <40% to AFM_1, AFM_2, AFB_{2a}, and AFG_{2a}. Table 1 shows a summary of these results.

Investigation of the Effects of Regeneration on Immobilized AFB_1—BSA Using ELISA. The previous results showing the use of NaOH and HCl solutions

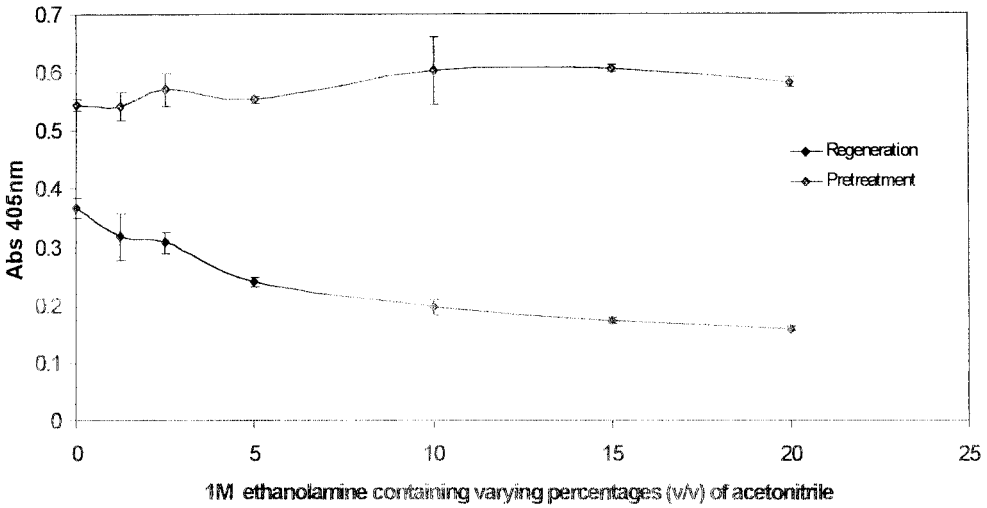

Figure 3. Effects of regeneration with 1 M ethanolamine, pH 12.0, containing various percentages (v/v) of acetonitrile on AFB_1−BSA coated onto wells of a 96-well microtiter plate. Increasing concentrations of the regeneration solution were added to coated wells before ("pretreatment") and after ("regeneration") incubation with antibody 2. The amount of bound antibody was measured by ELISA, with addition of HRP-labeled anti-rabbit IgG. Results shown are averages of five analyses.

for the regeneration of antibodies from AFB_1−BSA immobilized surfaces demonstrated that regeneration of the bound antibody from the surface caused the conjugate surface to be destroyed. As a result of this, a regeneration solution consisting of 1 M ethanolamine with 20% (v/v) acetonitrile, pH 12.0, was used. An ELISA was carried out to determine the suitability of this regeneration solution on an AFB_1−BSA surface.

AFB_1−BSA conjugate was coated onto wells of a microtiter plate, which were then blocked with 2% (w/v) milk; 100 μL/well of antibody 2 was then added to the wells, and incubation was carried out for 1 h at 37 °C. After washing, 100 μL of a range of 1 M ethanolamine solutions containing various percentages (v/v) of acetonitrile was added to appropriate wells, incubated for 10 min at room temperature, and then aspirated. Antibody 2 was then added, followed by HRP-labeled anti-rabbit antibody and a chromogenic substrate. The assay was then repeated with the regeneration solutions added to the plate after coating and before incubation with antibody. It could be seen that addition of increasing concentrations of acetonitrile (in the 1 M ethanolamine solutions) resulted in increased regeneration, whereas pretreatment of the conjugate surface did not significantly decrease the binding of polyclonal antibody at higher concentrations (Figure 3).

Preliminary Development of Antibody-Based Assay on BIAcore. After confirmation that antibody 2 recognized free toxin in ELISA, the development of an inhibitive SPR-based assay was evaluated. Nonspecific binding of the antibody preparation was examined. The AFB_1−BSA conjugate and BSA were immobilized onto separate sensor surfaces. There was negligible nonspecific binding of the antibody to the BSA protein (~5 RU). The antibody solution did not require preincubation with BSA, as all of the BSA binding antibodies were removed by subtractive immunoaffinity chromatography. It was found that preincubation of the sample with 100 μg mL^{-1} of CM-dextran) removed all nonspecific interactions with the CM-dextran surface. This was subsequently used in all antibody dilution preparations.

Efficiency of Regeneration. Antibody 2 gave an excellent binding response to AFB_1. A 1/100 dilution of the antibody was found to be sufficient to achieve significant binding to the immobilized conjugate surface

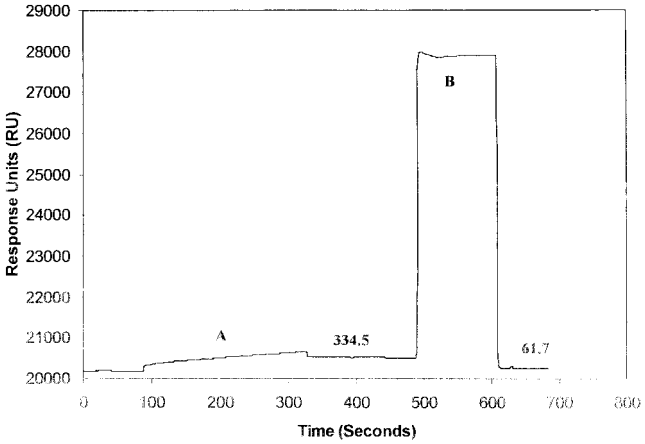

Figure 4. Sensorgram showing the binding of antibody 2 to the immobilized AFB_1−BSA surface: (A) 1/100 dilution of antibody was injected over the surface at 10 μL min^{-1} for 4 min, giving a response of 334.5 RU. The association curve suggested that antibody binding had not reached equilibrium after 4 min. (B) A 2-min pulse of 1 M ethanolamine with 20% (v/v) acetonitrile, pH 12, was required to remove the antibody from the surface of the chip. Although these were quite harsh conditions, all antibody was not fully removed from the surface each time. Approximately 61.7 RU remained on the surface after this regeneration.

(Figure 4). Initial studies on the sensor surface indicated that standard regeneration solutions such as NaOH and HCl were not sufficient to regenerate the bound antibody from the conjugate. As a result, a strong regeneration solution had to be employed consisting of 1 M ethanolamine with 20% (v/v) acetonitrile, pH 12.0. Although this solution was successful for regeneration of the surface, it did not remove all of the bound antibody each time. However, the accumulation of this bound antibody did not significantly affect binding over a large number of injections. The efficiency of the regeneration process was evaluated by performing multiple (i.e., 25) binding−regeneration cycles over the AFB_1−BSA-coated surfaces (Figure 5). The surface binding capacity of the antibody oscillated slightly over the 25 cycles, but it did not significantly affect the performance of the assay.

BIAcore Assay. An inhibition assay was then de-

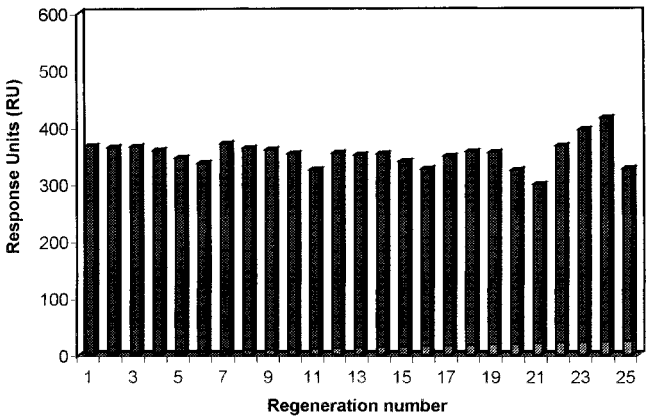

Figure 5. Graph showing the reproducibility of regeneration of the sensor chip with immobilized AFB_1−BSA on the surface. Twenty-five regenerations of antibody 2 were carried out using 1 M ethanolamine with 20% (v/v) acetonitrile, pH 12.0, as the regeneration solution. Approximately 15% of the antibody bound remained on the surface after each regeneration. It was not observed that this significantly affected the reproducibility of the binding.

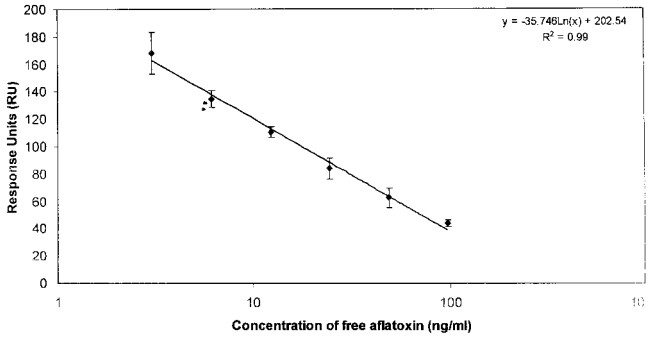

Figure 6. Inhibition BIAcore assay for determination of the optimal range of detection of free AFB_1. AFB_1−BSA conjugate was coated on the surface of the sensor chip at a concentration of 50 μg mL^{-1}, and the antibody dilution (antibody 2) was 1/100. A linear range of detection was obtained between 3.0 and 98.0 ng mL^{-1}. The R^2 value was found to be 0.99.

veloped by immobilizing 50 μg mL^{-1} of AFB_1−BSA conjugate prepared in 10 mM sodium acetate, pH 3.9. Standards of free aflatoxin were prepared ranging in concentration from 0.9 to 98.0 μg mL^{-1}. Each sample was incubated with an equal volume of a 1/50 dilution (to ensure a final dilution of 1/100) of antibody 2 for 10 min at room temperature and then passed over the surface of the chip, and this was carried out for each concentration in triplicate. The binding of antibody to the surface of the chip was inversely proportional to the amount of free aflatoxin in solution. Figure 6 shows the relationship between the number of response units bound and the concentration of free AFB_1. The intraday variability assay had a linear range of detection between 3.0 and 98.0 ng mL^{-1}. To carry this out, three sets of six standards were assayed on the same day, and their means were plotted. The R^2 value for this range was found to be 0.99. Interday variation was also carried out in which three sets of the five standards were run on three different days. The assay was reproducible over the 3 days between 3.0 and 49.0 ng mL^{-1}. Tables 2 and 3 show the coefficients of variation (CVs) for both sets of data. In contrast to ELISA (12.0−25000 ng mL^{-1}), the assay had a more sensitive linear range of detection.

Table 2. Intraday CVs for the BIAcore Aflatoxin B_1 Assay (Using Antibody 2)[a]

AFB_1 concn, ng mL^{-1}	calcd mean ± SD, RU	CV, %
97.65	43.700 ± 2.506	5.73
48.82	62.600 ± 7.188	11.48
24.41	84.030 ± 7.642	9.09
12.2	110.660 ± 3.963	3.58
6.1	134.900 ± 6.239	4.62
3.05	168.467 ± 15.236	9.04

[a] Three sets of six standards were run on the same day, and the CVs were calculated.

Table 3. Interday CVs for the BIAcore Aflatoxin B_1 Assay (Using Antibody 2)[a]

AFB_1 concn, ng mL^{-1}	calcd mean ± SD, A/A_0	CV, %
48.82	0.251 ± 0.024	9.561
24.41	0.342 ± 0.018	5.260
12.2	0.446 ± 0.025	5.610
6.1	0.561 ± 0.008	1.430
3.05	0.721 ± 0.018	2.500

[a] Three sets of five standards were run on three different days, and the CVs were calculated.

DISCUSSION

The results presented in this paper clearly indicate the lack of suitability of certain antibodies for use in a regenerable immunosensor and highlight some general considerations to be taken into account in the selection of the immunological component for such applications.

Both of the antibodies analyzed demonstrated a high avidity for the AFB_1−BSA conjugate, therefore making it difficult to regenerate surfaces. Immobilization of the anti-aflatoxin antibodies onto the sensor surface was previously attempted (Keating, 1998). This was carried out both directly and indirectly with the use of capture molecules such as antispecies antibodies and protein A. These strategies were unsuccessful. The direct immobilization of antibody onto the sensor surface resulted in the coupling chemistry significantly affecting antibody activity. Anti-species antibodies were also used, but no binding of the conjugate to the captured antibody could be observed. The use of protein A to noncovalently immobilize antibody was also examined. However, the dissociation of the polyclonal rabbit IgG from the protein A surface was significant, and no subsequent binding of the conjugate could be observed.

As the immobilization of antibodies had been shown to pose difficulties in terms of inactivation, and as strategies involving the use of capture molecules had proven to be unsuccessful, immunosensor formats based on the immobilization of the AFB_1−BSA conjugate were investigated.

The binding of antibody 1 to an immobilized AFB_1−BSA surface was examined. Although good binding signals were obtained (Figure 1), regeneration could not be achieved without affecting the antibody-binding capacity of the conjugate surface. Regeneration conditions, which were optimized for the removal of moderate levels of antibody 1, were found to be too harsh when only small amounts of polyclonal antiserum were bound, preventing the development of an inhibitive BIAcore immunoassay for AFB_1. Apart from a general indication of a high-avidity in the case of the anti-AFB_1 polyclonal antibody, no useful information could be gained from the BIAcore studies of this antibody preparation.

As it was not possible to develop a BIAcore assay with antibody 1, a polyclonal antibody (antibody 2) was produced. An inhibition ELISA was first developed with

the antibody to ensure that it recognized free toxin. The assay was found to be sensitive as well as reproducible (Figure 2). Initial studies with this antibody on BIAcore indicated that it was also difficult to remove after binding to the AFB_1−BSA conjugate surface using HCl and NaOH. Furthermore, it was not possible to completely regenerate the surface without reducing the binding capacity of the immobilized conjugate. ELISA was used as a method to investigate the effects of regeneration on immobilized conjugate. The use of a novel regeneration solution, consisting of 1 M ethanolamine with 20% (v/v) acetonitrile, pH 12.0, was assessed in ELISA. This solution combined high ionic strength and extreme pH as well as chaotrophic properties. It was found to be previously successful for use in surface regeneration of high-affinity molecules (Quinn et al., 1999). The studies showed that this regeneration solution did not significantly affect the integrity of the conjugate surface, and also that increasing concentrations of acetonitrile in the 1 M ethanolamine solution removed increasing amounts of antibody from the surface.

The combined properties of this solution enabled successful regeneration and consistent binding of antibody 2 to the AFB_1−BSA surface each time (Figure 5). Although there was consistent binding of the antibody after each injection, the regeneration solution did not appear to completely remove all of the bound antibody. These are probably "higher avidity" antibodies in the heterogeneous population of antibody, which are more difficult to regenerate. The use of high salt concentrations and organic solvents as regeneration solutions could also have been the cause of this antibody remaining, as contraction of the dextran gel can sometimes occur, giving rise to artifactual detector responses. It is significant for future biosensor applications that compatibility (even if limited) with organic solvents was demonstrated. Consequently, the possibilities for using small proportions of such solvents to aid the solubility of analytes will extend the range of contaminants that could be covered.

Nonspecific interactions between the antibody and the BSA part of the protein were minimal, as subtractive immunoaffinity chromatography (on a BSA column) was carried out on the antibody solution, which removed all BSA-binding antibodies. As a result, BSA did not have to be incorporated into the diluent buffer. The addition of CM-dextran to the diluent buffer ensured that there were no nonspecific interactions to the CM-dextran surface. A standard curve of the relative response against the AFB_1 concentration is shown in Figure 6. The limit of quantification of the assay was 3.0 ng mL^{-1}, and the assay was linear over the range 3.0−98.0 ng mL^{-1}, with a typical R^2 value of 0.99. Each sample was analyzed in triplicate and in random during all analyses. Intraday variability of the assay was assessed by running three sets of standards in one day and determining the CV between the calculated concentrations for each set of three. The CV values ranged from 3.58 to 11.48% and are shown in Table 2. Interday variability tests were also carried out, in which three sets of standards across the linear range were assayed on three different days. The CVs for these are shown in Table 2 and range from 1.43 to 9.56%.

Notwithstanding the limitations of the various capture systems (i.e., rapid dissociation and low levels of indirect immobilization), the main problem in developing a BIAcore inhibitive assay for AFB_1 was that of regeneration. The results obtained show that antibodies produced against AFB_1 have a high affinity for the conjugate. When analyzed on BIAcore, they display a high association rate and a low dissociation rate. These characteristics are not desirable if one wants to use the antibody in a regenerable biosensor. Anti-AFB_1 monoclonal antibodies, in particular, demonstrate high association rates and low dissociation rates (Keating, 1998). This may be due to the fact that the conjugate elicits a strong immune response and monoclonal antibody screening selects clones that give the highest responses in ELISA. These may have the highest affinities. Because a polyclonal antibody preparation is heterogeneous in nature, the antibodies can vary in affinities. Sometimes the majority of the antibodies in the preparation have a very high affinity (as in the case of antibody 1), and sometimes they can have moderate affinities, which can make them easier to regenerate (e.g., antibody 2).

One of the major difficulties with optical immunosensor systems is that of assessing how actual regeneration of antibody−antigen binding is taking place. Leeching of covalently bound material may be misinterpreted as a regeneration response, and the resulting decrease in detector response may be mistaken for complete regeneration of antibody−antigen binding. It is usually possible to correct for this effect by pretreatment of the immobilized surface with the regeneration reagents to be used, but this is not feasible when a novel interaction, for which the regeneration conditions are unknown, is being studied. In addition, the covalent bonds linking the immobilized component to the dextran gel may be disrupted by harsh chemical treatment, again resulting in a reduced signal. For example, the binding of 208 RU of antibody 1 to immobilized toxin−protein conjugate was almost completely regenerated by treatment of the surface with a 10-min injection of 100 mM NaOH. However, when only 27 RU of antibody bound to the surface, the same regeneration conditions brought the signal down to 111 RU below the baseline, suggesting that some immobilized conjugate had been removed. Clearly the availability of antibodies with suitable properties for use in the biosensor could be rate-limiting, especially as it has been shown that antibodies selected in ELISA and for ELISA are not necessarily suitable for use in the sensor. New approaches (as we have shown using the ELISA format) will be required for antibody selection.

CONCLUSION

Both of the antibodies examined bound specifically to AFB_1−BSA with a high avidity. This property is advantageous for use in ELISA, but it can hold limitations if the antibody is required for use in regenerable immunosensors.

The results presented show that microtiter plate-based ELISAs can be used to assist in the selection of antibodies and regeneration conditions for immobilized antigen surfaces. Although it is difficult to precisely relate the two formats in terms of contact time with the sensor surface, initial ELISA studies of this type can be used to identify the most effective type and concentration of regeneration reagents.

The use of organic solvents combined with high ionic strength was also assessed. This solution did not damage the conjugate surface, but it could not regenerate

all of the different antibodies. One antibody that was regenerated using this solution was antibody 2. Multiple regeneration cycles were performed on a conjugate coated surface, enabling the development of an SPR-based inhibitive immunoassay. The antibody showed a high level of sensitivity in the assay. The availability of an "on-line" antibody-based biosensor could have a significant impact on routine surveillance and analysis of agrifood materials.

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