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Biosensor-Based Screening Method for the Detection of Aflatoxins B_1-G_1

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Aflatoxins are extremely toxic metabolites from *Aspergillus* species that can adulterate a wide range of human foodstuff. Herein, we propose a novel assay designed as an analytical test for aflatoxin B_1 and G_1 (AFB_1 and AFG_1, respectively) that could represent an alternative screening technique for this class of mycotoxins. The approach for the determination of these toxins is based on surface plasmon resonance using neutrophil porcine elastase as a "bait" for these aflatoxins. The selection and optimization of the analytical procedure involved a preliminary investigation on the type of inhibition by AFB_1: the level of the protease inhibition exerted by AFB_1 depended upon the incubation time and the concentration of the binding partners, showing the competitiveness and the reversibility of the inhibition. A posteriori, the nature of the interaction granted a rapid analysis, a single detection test requiring only a few minutes. For the development of the assay, the experimental conditions were evaluated and optimized with both calibration solution and aflatoxin-spiked samples. To apply this method to aflatoxin-contaminated maize, a rapid solid-phase extraction treatment was developed. The proposed assay for AFB_1 and AFG_1 was validated by comparison with both a chromatographic reference method and a standard enzyme linked immunosorbent assay procedure. This enzyme-based biosensor represents a new approach for the detection of aflatoxins based on the reversible interaction between a blocked macromolecule and a soluble ligand, having the major advantages in the relative rapidity, the reusability of the capturing surface, and low cost per single test.

Mold can cause food contamination during different stages of production or storing. Aflatoxins are products of *Aspergillus* species and represent a major class of mycotoxins as assessed by their documented deleterious impact on both human and animal health[1,2] and consequently on economic aspects related to food commerce.[3] In this perspective, recent works provided insight into the acute effects of the aflatoxins on the gastrointestinal, respiratory, cardiovascular, and central nervous systems of both humans and animals.[4−6] Aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) are the most abundant of the aflatoxins in cereals and forages, and they have been considered the most toxic members of the aflatoxin family,[7−9] due to their "double-hazardous" ability to bind both DNA and proteins.[10−14] On such basis, a number of studies on aflatoxins have focused on their chronic carcinogenic, mutagenic, and hepatotoxic effects. Therefore, strict regulatory limits were established to protect both human and animal health and the food supply, although significantly differing from country to country.[15] Current reference methods are primarily chromatographic,[16−18] enzyme-linked immunoassay being a preliminary fast-screening method.[19−22] Whereas these have often shown the adequate sensitivity and specificity, all these assays presented

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some limits (need for specific—and expensive—antibodies, time-consuming analyses, etc.).

Therefore, given the demonstrated toxicity of aflatoxins, their potential widespread presence in the food chain, and the consequent economic wastes, much interest has been focused on the development of new practical screening assays[23−27] to be used for monitoring during the stages of food production, so that contaminated food can be properly labeled before feeding to animals or humans. Such methods should be rapid, sufficiently sensitive, and easily applicable, to allow the necessary corrective actions to be taken in a reasonable time. In this perspective, several rapid assays that do not require instrumental devices, such as immuno-based test strips,[28] quick flow-through assays,[29] and disposable columns,[30] have been recently developed.

In response to all these issues, surface plasmon resonance (SPR) based assays are considered as an alternative to conventional instrumental methods, to achieve faster and simpler detection of some environmental pollutants.[31−33] Concerning aflatoxins, the SPR biosensor approach is based on a macromolecule to be used as a capturing agent of the analytes, namely, AFB_1 and AFG_1. In particular, our preliminary investigations showed the competitive inhibition of a major serine protease, namely, neutrophil elastase, by AFB_1. On such basis, herein we propose a method to detect these two aflatoxins in the low parts per billion range by following the increase in SPR response upon addition of soluble aflatoxins to an elastase-derivatized biosensor.

EXPERIMENTAL SECTION

Caution Note. Solid aflatoxins are tremendously hazardous. Extreme care was exercised in aflatoxin handling, including gloves, respiratory masks, and chemical fume hoods. Aflatoxin contaminated surfaces, glassware, and disposables were treated with 20% NaOCl before reuse or disposal. Maize samples spiked (or contaminated) with aflatoxin were unambiguously labeled, kept in a dedicated chemical fume hood, and placed in biohazard safety bags prior to proper disposal.

Reagents, Chemicals, and Devices. Porcine neutrophil elastase, succinyl-Ala-Ala-Ala-4-nitroanilide, Tween-20, AFB_1, AFB_2, AFG_1, AFG_2, and fumonisin B_1 were purchased from Sigma-Aldrich. AFM_1, ochratoxin A, deoxynivalenol, zearalenone, α- and β-endosulfan, 1,1,1-trichloro-2-(2-chlorophenyl)-2-(4-chlorophenyl)ethane (*o*,*p*′-DDT), 1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane (*p*,*p*′-DDT), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (*p*,*p*′-DDD), and 1,1-dichloro-2,2-bis(4-chlorophenyl)ethene (*p*,*p*′-DDE) were kindly provided by the Istituto Zooprofilattico Sperimentale di Umbria-Marche (Italy). NaOH, NaCl, Tris, KCl, NaH_2PO_4, Na_2HPO_4, KH_2PO_4, MeOH, EtOH, TFA, *n*-hexane, and CH_3CN were obtained from J.T.Baker (Netherlands). All chemicals were of the highest grade available. Carboxylate functionalized cuvettes and the EDC-NHS immobilization kit were obtained from Neosensors (Crewe, UK). Solid-phase extraction C-18 Sep-Pak Plus cartridges were purchased from Waters Italia (Italy). Inhibition studies were performed on a Cary10 spectrophotometer, Varian (California, USA). Binding experiments were performed on an IAsys plus device, Affinity Sensors (Cambridge, UK), obtained from ThermoFisher Scientific (Waltham MA, USA). ELISA Ridascreen Aflatoxin-Total and RIDA Aflatoxin immunoaffinity columns were obtained from R-Biopharm AG (Germany). ELISA plate readings were performed on a BioTrak device obtained from Amersham-Bioscience (UK). Phenomenex Luna HPLC RP-C18 column (250 × 3.0 mm; 5 μm) and C18 Security Guard cartridge (2 × 4 mm, 2 μm) were obtained from Phenomenex (Torrace CA, USA). The HPLC spectra system, constituted by a P4000 quaternary HPLC pump, a SN4000 system interface, a SCM1000 solvent degasser system, a FL3000 fluorescence detector, and an AS3000 autosampler, was obtained from Thermo Fisher Scientific (Waltham MA, USA). The 7010-151 injection valve was obtained from Rheodyne (Cotati CA, USA). The Jones Chromatography column heater (7990R-1-L) was provided by STEPBIO (Bologna, Italy). Aflatoxin stock solutions were prepared in methanol, and their concentration was determined spectrophotometrically according to the official procedure.[34]

Maize samples: AFB_1-contaminated maize was provided by Istituto Zooprofilattico Sperimentale Umbria-Marche (Perugia, Italy), and the aflatoxin-free certified T400A maize sample was obtained from Central Science Laboratory (Sand Hutton York, UK).

Measure of Aflatoxin Effect on Elastase Activity. The equilibrium dissociation constants of the elastase−aflatoxin complex were derived from the measure of the inhibitory effect on the amidolytic activity toward succinyl-Ala-Ala-Ala-4-nitroanilide. AFB_1 was used as a model of aflatoxin in this preliminary assay. The enzyme was preincubated for 10 min at 25 °C with increasing concentrations of AFB_1 in a 50 mM phosphate buffer, pH 7.5 at 25 °C (longer preincubation times did not further affect residual activities). The reaction was started by addition of the substrate. Residual activities were measured at 410 nm, as the ratio of the initial velocity of the product formation in the absence (V_o) and presence ($V_{o,i}$) of a given AFB_1 concentration $[I]_i$.

$$a_i = \frac{V_{o,i}}{V_o} \tag{1}$$

The experimental data set was constituted by a set of residual activities a_i measured at increasing AFB_1 concentrations, each repeated at four different substrate concentrations, in the range 1−40 μM. The apparent dissociation constant ($K_{D,app}$) for each

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substrate concentration [S_0] was derived from the standard equation for tight binding inhibitors of proteolytic enzymes (eq 2) to be used upon the addition of a limited amount of the tight-binding ligand, i.e., when almost all the inhibitor is in the combined form ([E_t] ≥ $K_{D,app}$[35,36])

$$a_i = 1 - \frac{([E_t] + [I]_i + K_{D,app}) - \sqrt{([E_t] + [I]_i + K_{D,app})^2 - 4[E_t][I]_i}}{2[E_t]} \quad (2)$$

where [E_t] is the total concentration of the enzyme (both free and inhibitor- and/or substrate-bound) and [I] is the concentration of the inhibitor.

The relationship between apparent and real equilibrium dissociation constants can be expressed by eq 3

$$K_D = \frac{K_{D,app}}{\left(1 + \frac{[S_0]}{K_m}\right)} \quad (3)$$

where K_m is the concentration of substrate that leads to half-maximal velocity.

Binding of AFB_1 and AFG_1 to the Elastase-Functionalized Sensor Surface. The sensing chamber was thermostatted at 25 °C. The surface was washed and equilibrated with PBS buffer, then activated by addition of an equimolar mixture of *N*-hydroxysuccinimide and *N*-ethyl-*N*-(dimethylaminopropyl) carbodiimide hydrochloride.[37] The interactant to be immobilized was dissolved in 10 mM acetate buffer, pH = 6, and incubated over the surface for 10 min. Unreacted carboxylic sites on the sensor surface were deactivated by injection of 1 M ethanolamine, pH 8.5. The surface was finally re-equilibrated with PBS. AFB_1 and AFG_1 reference standards were added at different concentrations, and association kinetics were followed up to equilibrium. Dissociation steps and surface regeneration were performed by addition of fresh buffer, each time assessing the baseline recovery prior to any further addition of aflatoxin. Raw data were globally fitted to a monophasic model

$$R_t = R_{eq,[L]}(1 - e^{-(k_{ass}[L] + k_{diss})t}) \quad (4)$$

where R_t, the response at equilibrium, is

$$R_{eq,[L]} = \frac{R_{max}k_{ass}[L]}{k_{ass}[L] + k_{diss}} \quad (5)$$

and R_{max} is the extent at asymptotically high concentrations of [L]. Time courses measured at several ligand concentrations can be simultaneously analyzed using eqs 4 and 5, sharing common k_{ass}, k_{diss}, and R_{max} parameters.

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Sample Collection and Preparation. Uncontaminated maize samples were crushed to powder using a high-speed benchtop blender, and then 4 g was spiked with 200 μL of AFB_1 solution at different concentrations. Spiked samples were mixed and extracted according to AOAC method n.991.31 by stirring with 10 mL of 70% methanol, then filtered with Bibby-Sterilin filter paper to eliminate insoluble materials. The filtered solution was cleaned up from possible interfering agents (and secondarily) preconcentrated by solid-phase extraction using Waters C18 Sep-Pak Plus cartridges. Blank and contaminated samples were treated according to the same protocols.

Sample Cleanup. The C18 Sep-Pak Plus cartridge was connected to a peristaltic pump: the optimal flow rate was measured to be 0.5 mL/min (the flow rate was controlled by measuring outlet volumes at a fixed time). Previously, FEP-tygon tubings and PTFE connectors were conditioned with methanol. The cartridge was primed with 20 mL of distilled water and activated with 10 mL of methanol. Next, the aflatoxin spiked samples (10 mL) were loaded on the C18 cartridge and eluted by successive washes of 20, 40, 60, and 80% methanol (5 mL each wash). Polar compounds were previously eluted with 5 mL of NaCl 0.2 M. All fractions were collected in silanized vials and tested by HPLC and ELISA. AFB_1 was eluted with 80% methanol. The cartridge was finally regenerated with 20 mL of 100% methanol.

ELISA Test. The Ridascreen Aflatoxin ELISA test preliminarily screened the presence and the recovery of the toxin in the different fractions. Spiked solutions, standards, and blank samples (50 μL) were added to reference wells. Proper dilution of peroxidase conjugated aflatoxin and antiafla mouse monoclonal antibodies solution were added to each well, carefully mixed, and incubated for 30 min at room temperature in the dark. The mixtures were removed, and the wells were gently rinsed three times with distilled water. Then, equal volumes of urea peroxide and tetramethyl-benzidine (50 μL) were added to each well and incubated for 30 min at room temperature in the dark. The reaction was stopped by addition of 1 M sulfuric acid. Absorbances were read at 450 nm, within 60 min after the addition of the stopping solution. Each sample was analyzed in triplicate.

HPLC Assay. The biosensor-based assay was eventually validated according to a slight modification of the official AOAC HPLC method n.990.33. Both AFB_1-spiked samples and blank samples were extracted with methanol−water (70:30 v/v) and purified with RIDA Aflatoxin immunoaffinity columns, then dried by a gentle stream of nitrogen. Dried samples were derivatized by TFA to enhance AFB_1 intrinsic fluorescence. AFB_1 concentrations were finally assessed by HPLC with fluorescence detection, using a Phenomenex Luna C18 column equipped with a C18 Security Guard cartridge. The column was thermostatted at 30 °C using a Jones column heater. The mobile phase was a mixture of $CH_3CN/MeOH/H_2O$ (17/29/54, v/v/v) maintained at a flow rate of 0.34 mL/min, and the separation was carried out in isocratic conditions. Aflatoxin was detected using a fluorescent detector (λ_{ex} = 364 nm, λ_{em} = 434 nm).

Aflatoxin Detection in Maize Extracts by Biosensor. Different dilutions of aflatoxin-containing and aflatoxin-free fractions were added to the elastase-functionalized surface, and each response kinetic was routinely followed and analyzed as described above. The regeneration of the elastase monolayer was carried

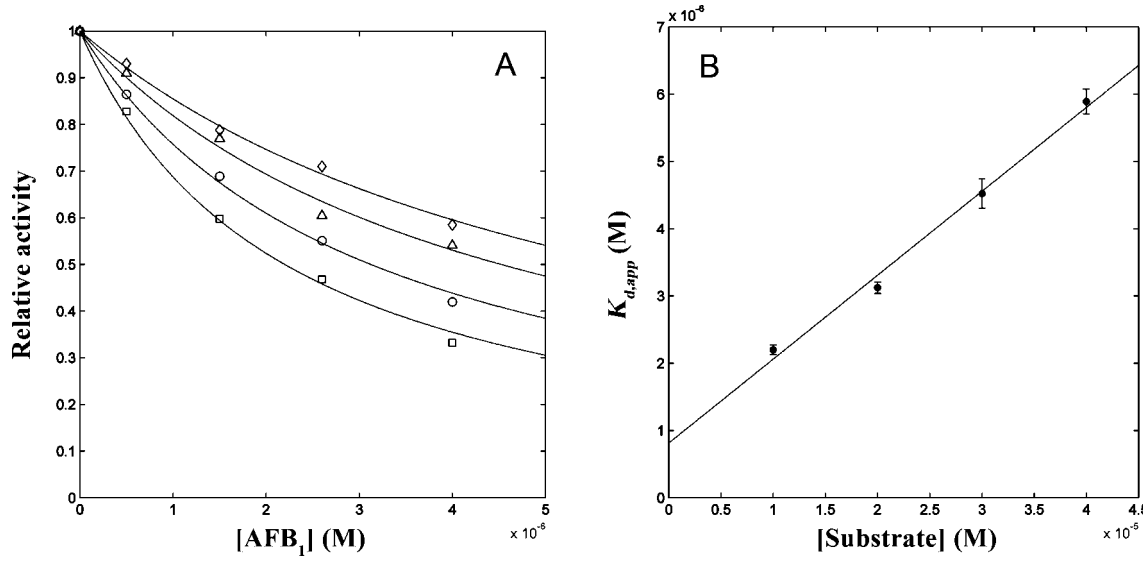

Figure 1. Inhibitory effect on the amidolytic activity of elastase by AFB_1. Elastase relative activity versus AFB_1 concentration (inset A) measured at four different substrate concentrations: 500 μM (◇), 50 μM (Δ), 5 μM (○), 0.5 μM (□). All determinations were carried out in 50 mM phosphate buffer, pH 7.5 at 25 °C. $K_{D,app}$ versus substrate concentration: linear regression of K_D (inset B).

out as previously described. Detection procedures were replicated on different days on both the same and different elastase-functionalized surfaces. Additionally, the assessment of the number of regeneration cycles that a sensor surface can withstand without a significant loss of the sensitivity and accuracy of the assay and the stability of the sensing surface throughout multiple measurements were evaluated.

Limits of Detection and Quantitation. In compliance with the IUPAC rules,[38] the limit of detection (LOD) was calculated as three times the standard deviation of the blank measurements. The limit of quantification (LOQ) is calculated as 10 times the standard deviation of the blank measurements. Aflatoxin-free certified T400A maize sample was used as a blank matrix.

Cross-Reactivity. To evaluate the selectivity of the proposed assay, equilibrium dissociation constant values (K_D) were determined using other toxins (AFB_2, AFG_2, AFM_1, fumonisin B_1, ochratoxin A, deoxynivalenol, and zearalenone). Different dilutions of (afla)toxin standard solutions were added to the elastase-functionalized surface and routinely analyzed as described above.

Cross-reactivity values were calculated as

$$CR\% = \frac{[AFG_1]_{R_{max}/2}}{[AFx]_{R_{max}/2}} \times 100 \qquad (6)$$

where R_{max} corresponds to the maximum response at equilibrium obtained for AFG_1, and $[AFG_1]_{R_{max}}/2$ is the concentration of AFG_1 yielding the half-maximal response.

Moreover, the elastase-based biosensor selectivity was additionally tested toward other common maize contaminants (α- and β-endosulfan, *o,p′*-DDT, *p,p′*-DDT, *p,p′*-DDD, and *p,p′*-DDE).

RESULTS AND DISCUSSION

Inhibition Studies. The interaction between elastase and AFB_1 was assessed using a standard spectrophotometric inhibition assay. Amydolitic activities toward the respective chromogenic substrate in the presence of increasing amounts of AFB_1 were all characterized by hyperbolic inhibition isotherms, whose steepness decreased at increasing substrate concentrations (Figure 1).

Inhibition data were fitted using a 1:1 stoichiometry model for a reversible competitive inhibitor (eq 2), as earlier reported for serine proteases binding to small molecule inhibitors;[39] the competitive nature of the inhibition was confirmed by the Dixon plot analysis (data not shown). $K_{D,app}$ values, the substrate-dependent equilibrium dissociation constants of the serine protease−ligand complex, were obtained for each substrate concentration: $K_{D,app}$ was linearly dependent upon the substrate concentration (Figure 1, inset B), consistently with the competitive inhibition hypothesis, thus the real K_D was extrapolated to zero substrate concentration ($K_D = 0.8 \pm 0.2$ μM).

Binding Studies. The binding between AFB_1 and AFG_1 and tethered elastase was tested (Figure 2). The binding surface containing immobilized serine proteases was obtained as described in the Experimental Section. A shift in sensor response ($\Delta R = 800$ arcseconds) upon elastase immobilization was reported, corresponding to a final surface concentration of 1.7 ng/mm^2 (approximately equivalent to 7 mg/mL). The immobilization protocol was optimized after several experiments based on the variation of elastase concentration and immobilization pH value. An elastase concentration value of 100 μg/mL and a value of 6 for the immobilization pH (based on elastase isoelectric point) allowed an optimal immobilization of elastase together with keeping its functional properties. Local and global fit analysis of the interaction data revealed monophasic kinetics. Furthermore, monoexponential analysis of association curves residuals was not affected by measurable systematic errors (a biexponential model did not considerably improve the quality of the fit as judged by an F-test, 95% confidence). Equilibrium dissociation constants were 0.91 ± 0.03 μM and 0.69 ± 0.16 μM, respectively for AFB_1 and

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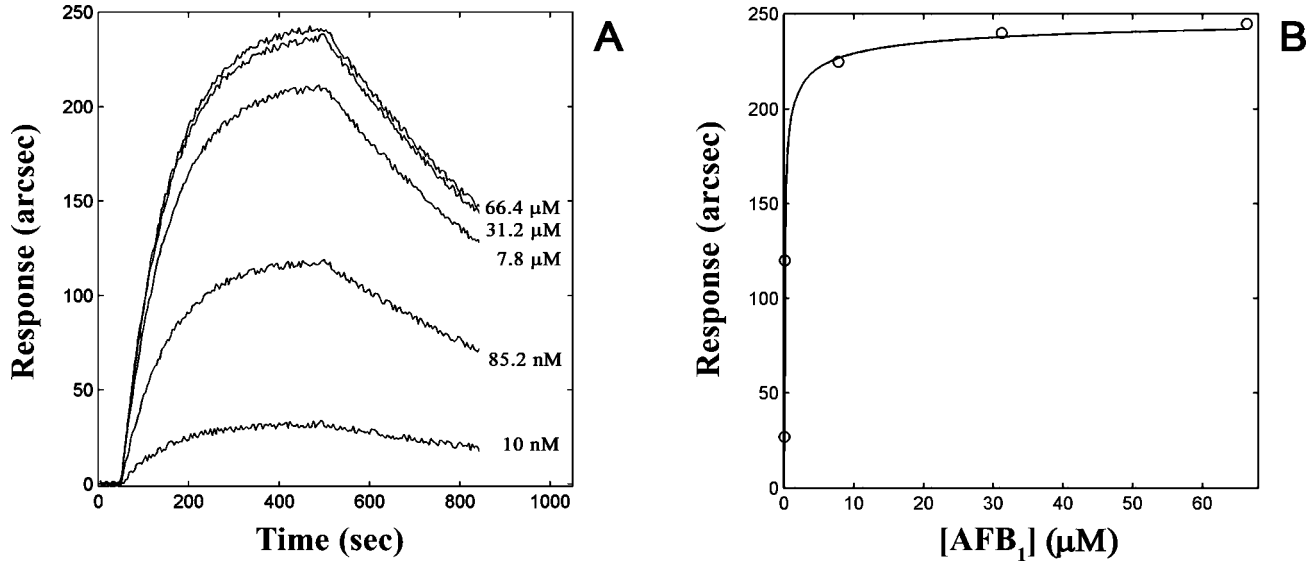

Figure 2. Binding of AFB_1 to immobilized elastase. Association and dissociation curves overlay measured at increasing concentrations of AFB_1 (inset A). Extent of binding (inset B).

AFG_1. Therefore, since the assay detected AFB_1 and AFG_1 with comparable sensitivity, AFB_1 was chosen to generate the calibration curve.

Moreover, measured K_D obtained both by spectrophotometric assay ($K_{D,S}$) and by biosensor studies ($K_{D,B}$) resulted in comparable values, confirming a similar functional behavior for serine protease−aflatoxin complexes both in solution and as blocked onto the biosensor surface. Moreover, calculated K_D values and the asymptotic saturation reached at asymptotically high aflatoxin concentrations (Figure 2, inset B) confirmed a specific binding site on the enzyme for the aflatoxins. The analysis of association (k_{ass}) and dissociation (k_{diss}) rate constants for AFB_1 and AFG_1 binding to elastase allowed an insightful understanding of the mechanistic properties of the macromolecular recognition process. In particular, kinetic association rates were 12900 ± 700 $M^{-1}s^{-1}$ and 13060 ± 1060 $M^{-1}s^{-1}$, while kinetic dissociation rates were 0.011 ± 0.001 and 0.009 ± 0.002 s^{-1}, respectively. Both association and dissociation rates are consistent with previous studies on the kinetics of serine proteases binding to other small ligands.[39,40]

Optimization of the Biosensor Assay. To develop an efficient biosensor-based assay for AFB_1 and AFG_1 determination, the experimental conditions had to be properly considered and optimized, taking into account parameters such as enzyme concentration, immobilization pH, temperature, etc. An enzyme concentration of 100 μg/mL was chosen because it allowed the achievement of a reasonably high surface density: in fact, an excessive surface density could hinder the accessibility of the analyte to the binding site of the enzyme, thus reducing the number of free binding sites on the sensing surface and impairing the determination. Under these conditions, the determination of AFB_1 and AFG_1 at subsaturating concentrations ([AFLA] < K_D) assured a good reproducibility of the assay on different elastase-functionalized surfaces. Besides, the reversibility of the interaction permitted the achievement of an unambiguous response upon AFB_1 and AFG_1 binding to elastase within 2 min.

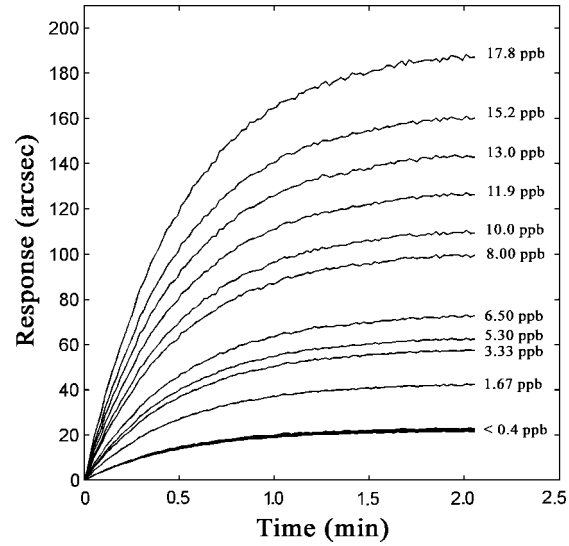

Figure 3. Biosensor screening analysis. Overlay of association kinetics upon addition of aflatoxin B_1 contaminated extracts in the linearity range 1.67−17.8 μg/kg. Responses of replicate additions of aflatoxin-free certified T400A maize ([Afla] < 0.4 ppb) are included.

Calibration Curve. Biosensor-based assay was applied for the determination of AFB_1 using spiked maize samples (Figure 3). The analysis of the binding of AFB_1 to elastase over the concentration range 1−50 μg/kg (data not shown) reported that the response for the optimized assay was linear in the range between 1.67 and 17.8 μg/kg (Figure 4). Each day, multiple calibration curves ($m = 4$) were performed by adding ten aflatoxin concentrations ($n = 10$), and the results showed a linear correlation: $y = (9.2 \pm 0.8)x + (22 \pm 8)$, where y is the response upon addition of AFB_1 and x is AFB_1 concentration, R^2 being equal to 0.988. This calibration procedure was replicated on three different days. The experimentally measured lower limit of the linear range was 1.67 μg/kg of AFB_1, whereas the K_D was 0.91 μM (≈250 μg/

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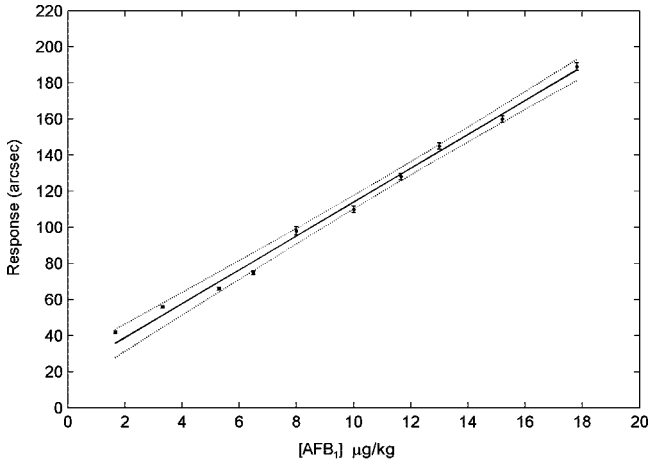

Figure 4. AFB_1 calibration curve. Linear fit (solid line) and 95% confidence bound (dashed lines) are reported. Each experimental point was the average of four replicates.

Table 1. Comparison of Recovery Studies of Spiked Maize Samples AFB_1[a]

	ELISA		HPLC		BIOSENSOR	
	70% MeOH extraction		70% MeOH extraction and immunoaffinity cleanup		70% MeOH extraction and SPE	
added	found	% recovery ± SD	found	% recovery ± SD	found	% recovery ± SD
9.5	7.58	80 ± 3	7.03	74 ± 2	7.52	79 ± 2
16	12.69	79 ± 5	11.84	74 ± 1	12.67	79 ± 3
24	19.30	80 ± 3	17.52	73 ± 2	19.40	81 ± 2

[a] Concentrations are expressed as µg/kg.

kg) AFB_1. The detection limits reached allow us to use this assay for detection of AFB_1 in maize within the regulatory limits.[41]

AFB_1 Measurement in Fortified Maize Samples. Methanol had a vanishable effect on elastase activity (data not shown), whereas a significant effect was measured for the biosensor determination, due to the variation in refractive index of the solution dictated by the addition of methanol to an aqueous solution (see Figure 3).

First, different dilutions of the sample in buffer were evaluated to obtain the lowest matrix and solvent effects and to decrease the response of samples within the linearity range. The response of certified blank maize samples upon the addition of 1/15 sample dilution in PBS buffer showed the lowest matrix and solvent effects. This dilution factor was adopted during the study.

The extraction efficiency of the toxin from maize was determined using samples spiked before the extraction with known amounts of the toxin.[24] Different aliquots of AFB_1 standard solution were added to 4 g of blank maize to obtain 9.5, 16, and 24 µg/kg of maize sample. For each AFB_1 level, three samples were independently processed, and each sample was analyzed in triplicate. On the basis of the calibration curves prepared in maize extract, it was possible to calculate the extraction efficiency of the analyte.

The results show that the procedure adopted for extraction of AFB_1 from maize is relatively quick (10 min) with a recovery of 80%. In Table 1, we compared the recoveries of the three different detection methods. Specifically, the SPE treatment did not affect the recovery, being characterized by a 100 ± 3% recovery, as measured evaluating AFB_1 content before and after the solid-phase extraction by HPLC. Interestingly, a biosensor assay showed a comparable total recovery (80%). An advantage of this method is that it is faster than reference methods such as HPLC,[16−18] and moreover, it uses less expensive reagents than the specific antibodies adopted in ELISA.[19−22]

Table 2. Equilibrium and Kinetic Constants Determined for Different Toxin−Elastase Complexes[a]

	K_D (µM)	k_{ass} ($M^{-1}s^{-1}$)	k_{diss} (s^{-1})	CR ratio (%)
AFB_1	0.90 ± 0.03	12900 ± 700	0.011 ± 0.001	84.3
AFB_2	3.61 ± 1.21	8310 ± 220	0.03 ± 0.01	21.5
AFG_1	0.69 ± 0.16	13060 ± 1060	0.009 ± 0.002	100
AFG_2	4.77 ± 0.99	11105 ± 305	0.05 ± 0.01	1.1
AFM_1	1.25 ± 0.37	13600 ± 670	0.017 ± 0.005	66.3

[a] The data reported in the table represent the average of three measurements.

Table 3. Comparison of the LOD, LOQ, and Linearity Range of a Selection of Different Methods for the Analysis of Aflatoxins

method	LOD (µg/kg)	LOQ (µg/kg)	linearity range (µg/kg)
LC-MS[43]	10	25	10−600
LC-MS/MS[44]	0.09−0.51	0.27−1.50	−
MALDI-TOF MS[45]	0.0005	−	−
HPLC fluorescence detector[17]	3.5	4	4−20
HPLC amperometric detector[46]	7−10	−	−
TLC densitometric analysis[47]	1.2−1.7	1.9−2.8	1−9
IMS[48]	0.1	0.5	1−10
UV−vis enzymatic method[24]	10	−	10−60
this method	0.97	3.10	1.67−17.8

Cross-Reactivity. Possible cross-reactivity with other (afla) toxins or maize contaminants was evaluated. As summarized in Table 2, AFB_1, AFG_1, and AFM_1 targeted elastase with comparable affinity, whereas both AFB_2 and AFG_2 showed a 5-fold lower ability to bind/inhibit the enzyme. This different behavior can be partly attributable to the presence/absence of the double bond at the dihydrofuran moiety, this difference relevantly affecting the binding of the toxin within the catalytic site of the enzyme, as suggested by predictive complexes obtained by computational binding analysis (data not shown). The obtained results showed that the assay can detect AFB_1, AFG_1, and AFM_1 with comparable sensitivity.

Additionally, to better understand the behavior of the assay in the case of an AFB_1−AFG_1 mixture (since AFM_1 is not a maize contaminant), the two toxins were analyzed both individually and as a mixture. AFB_1 was added at two different concentrations (6.5 and 13 ppb), and the responses were recorded (75 and 145 arcsec, respectively). The same experiments were performed using AFG_1, obtaining a comparable response (80 and 153 arcsec, respectively). Then the response upon the addition of a mixture of aflatoxins (6.5 ppb AFB_1 and 6.5 ppb AFG_1) was measured, with a resulting 149 arcsec response, confirming the ability of the method to detect AFB_1 and AFG_1 with similar sensitivity.

Conversely, other toxins and pesticides tested (α- and β-endosulfan, *o*,*p*′-DDT, *p*,*p*′-DDT, *p*,*p*′-DDD, and *p*,*p*′-DDE) did not show any specific binding to elastase.

Reusability and Efficiency of the Sensor. The effect of different regeneration agents on the sensor reusability was studied. The dissociation of the aflatoxin/elastase complexes was performed by washes with both acidic and neutral buffer solutions (the PBS binding buffer). Using 10 mM HCl, the sensor surface could be used without loss of activity for 20 measurement cycles before any loss of binding capacity was reported. Nevertheless, the sensing surface resisted to a higher number of experimental cycles when the buffer solution was used (biosensor response did not change more than 5% after 50 regeneration cycles). Consequently, the use of the buffer solution provided an efficient (even if slower) desorption of the ligand without degrading the immobilized enzyme. The assay reproducibility was dissected by comparison of the intra- and interday variability. To assess the daily variation, the calibration curves were repeated on different days, and the maximum intra- and interday coefficients of variation (3.48% at 8 ppb, and 5.78% at 3.33 ppb, respectively) were within the confidence limits of the calibration curve. Additionally, the "surface-to-surface" variation of assay sensitivity was evaluated on different elastase-derivatized biosensors. This variation was negligible, with K_D values ranging within the standard deviation, independently from the aflatoxin or elastase stocks used.

CONCLUSIONS

We developed an enzyme-biosensor method for the detection of AFB_1 and AFG_1. The determination is based on their ability to specifically bind neutrophil porcine elastase, and it makes use of a biosensor-based method to measure the response upon the binding of the analyte. According to the proposed method, within a few minutes (2 min for the biosensor analysis), it is possible to obtain results suitable for rapid screening of the prepared samples (requiring 10 min for the extraction and 30 min for the SPE cleanup). A detailed investigation of the kinetics and inhibitory mechanism using AFB_1 specified a competitive type. In particular, the high kinetic association rate contributed to increase the velocity of the response of the instrument.

Globally, the proposed method presented some major advantages with respect to ELISA and HPLC tests, such as the minimal consumption of (low expensive) reagents, a higher quickness of the test, together with maintaining comparable specificity and sensitivity.

Cross-reactivity data showed that elastase is able to analytically discriminate between AFx_1 and AFx_2 (Table 2); nevertheless, the low specificity within the AFx_1 class does not necessarily reduce the value of the assay as a screening method for aflatoxin detection in foodstuff, since AFM_1 does not represent an interfering agent for monitoring of cereals, being present only in milk and milk derivatives.[42] LOD and LOQ for AFB_1 (determined as described in the Experimental Section) were calculated to be 0.97 and 3.10 μg/kg, respectively, making this assay a useful tool for the screening of aflatoxins in food. Moreover, LOD, LOQ, and linearity range of the proposed method are comparable to those available for other analytical approaches (Table 3).

Future works will be focused on reaching a lower LOQ (<2 μg/kg) according to current European regulation for analysis of aflatoxin in human food[41] and on the improvement of the specificity of the method.

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