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RESEARCH ARTICLE

Fluorescent competitive aptasensor for detection of aflatoxin B_1

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Abstract

Aflatoxin B1 (AFB_1) is one of the most commonly found mycotoxins in food commodities, particularly cereals, oilseeds, spices and tree nuts. In the past decade, aptamers have come into limelight and emerged as a new biosensing element replacing antibodies in various detection formats. Herein we report a faster, more sensitive, high throughput method for the detection of AFB_1 using AFB_1-specific aptamers. The assay format was based on a competitive reaction of the fluorescent tagged aptamer specific to AFB_1 with the aflatoxin conjugate. Under optimal conditions, a linear range of detection (50 ng to 50 pg) was achieved with a limit of detection (LOD) of 10 pg/mL in the buffer system. Results of inter- and intra-assay revealed that the assay was repeatable with standard deviation in acceptable range. The assay was also validated in food samples such as dried red chilies, groundnut and whole pepper with recovery in the range of 92 to 102% at 10 ng/mL and 100 pg/mL levels. The aptasensor assay was also compared with standard analytical method of HPLC and was found to be more sensitive. This detection technique has the potential to be developed into a biosensor platform for AFB_1 detection.

KEYWORDS

Aflatoxin B_1, aptasensor, competitive binding, fluorescence, food samples

1 | INTRODUCTION

Aflatoxin B_1 (AFB_1), a toxic secondary metabolite produced mainly by molds like *Aspergillus flavus* and *Aspergillus parasiticus*, possesses the highest toxicity among all the aflatoxins. AFB_1 has been listed as a group I carcinogen by International Agency for Research on Cancer.[1] Various regulatory bodies have set permissible limits for AFB_1 in food and feedstuff. The US Food and Drug Administration have stated an action level of 20 ppb of AFB_1 in food,[2] whereas the European Union has set a limit of 2 μg/kg of AFB_1 in all cereal and cereal products.[3] AFB_1 has been detected in ppb levels in food and agricultural commodities like cereals, spices, groundnuts, wine and feedstuff, thus making its detection crucial.

Conventional instrumental methods like high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS)[4,5] are used for quantification of AFB_1, but these methods require trained personnel for operation, have costly instrumentation and are time consuming. Alternatively, ELISA-based kits are commercially available along with various reports on immunosensors for the detection of AFB_1.[6] However, the use of antibodies restrains them in terms of time consumption for antibody generation, production cost, storage stability, susceptibility for cross-reactivity, false-positive or false-negative results and matrix interference effect.

In the recent years, aptamers have emerged as a new biosensing element for detection of analytes ranging from ions to cells.[7,8] Aptamers are short, single-stranded oligonucleotide sequences or peptide sequences generated against target molecules *in vitro*. They can be easily screened, evolved and designed by an *in vitro* selection process known as systematic evolution of ligands by exponential enrichment (SELEX). Aptamers fold into specific 3-dimensional structures, which result in their target identification. They bind to their ligands through complementary shape interactions and can incorporate small molecules into their nucleic acid structure.[9] Aptamers offer advantages over antibodies like low cost, better stability and robust application in various analytical methods.

Aptamers have been selected against AFB1 using magnetic nanoparticle-based SELEX[10] and monoclonal surface display SELEX.[11] Neoventures Biotechnology, a Canadian company, has developed AFB_1-specific aptamers and also holds a patent for it.[12] Recently, many aptamer-based biosensing methods have evolved for AFB_1 detection

where electrochemical,[13] quantum dot-graphene oxide-based fluorescence quenching,[14] HRP-DNAzyme,[15] aptamer-based dipstick assay[16] and real-time quantitative PCR (RT-qPCR)[17]-based aptasensor have been reported. Although these methods are efficient, ultrasensitive and promise on-site detection, the use of antibodies, associated toxicity, costly instrumentation and complicated assembly limit their application.

In this study, we attempt to develop a platform for the detection of AFB_1 using AFB_1-specific aptamer. The assay was validated by analyzing dried red chili, pepper and groundnut samples spiked with known concentrations of AFB_1.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

All analytical-grade chemicals and solvents were purchased from Himedia Laboratories (Mumbai, India) and SRL (Mumbai, India). AFB_1 aptamer 5′ TTGGGCACGTGTTGTCTCTCTGTGTCTCGTGCCC TTCGCTAGGCCCACA3′)[18] was modified at the 5′ end with a fluorophore (6-FAM). AFB_1-BSA coating antigen and AFB_2, AFG_1 and AFG_2 standards for specificity studies were procured from Sigma-Aldrich (Bangalore, India). AFB_1 standard was obtained from Himedia Laboratories (Mumbai, India). All AFB_1 standards and aptamer dilutions were made in 1:1 PBS–diethanolamine buffer. The buffers were prepared using double distilled water.

2.2 | AFB_1-protein conjugates

AFB_1-protein conjugate (AFB_1-BSA), which was used as coating antigen in this study, was prepared by first derivatizing AFB_1 into AFB_1-oxime according to the method previously described.[19] Carrier protein (BSA) was then attached to AFB_1 using the activated ester method.[20]

2.3 | Optimization of the assay

The aptamer assay developed in this study was based on similar procedures pertaining to ELISA except that there was no antibody used. The BSA-conjugated AFB_1 hapten was immobilized onto the black 96-well immune plates (ThermoScientific, Bangalore, India) overnight at 4°C. The conjugate was washed with PBS-Tween 20 (PBS-T) to remove the unconjugated hapten. The vacant sites were then blocked with 1% gelatin for 2 hours. After washing with PBS-T, the analyte AFB_1 was added and incubated at 37°C for 1 hour. AFB_1 aptamer (0.1μM) was added to the wells, and the incubation was continued for another hour. Aptamer addition to hapten conjugate without the addition of AFB1 served as the control. After a PBS-Tween 20 wash, the reactants were suspended in PBS buffer for measuring fluorescence. Fluorescence of the samples were measured at an excitation wavelength of 494 nm and emission wavelength of 515 nm.

All fluorescent measurements were analyzed using Varioskan Flash microplate reader (Thermoscientific) and were carried out in replicates to determine the assay precision.

2.4 | Validation of the aptamer assay

The optimized aptamer assay was validated by testing its sensitivity using various concentrations of AFB_1 (100 ng, 50 ng, 25 ng, 10 ng, 100 pg, 50 pg, 10 pg and 100 fg) prepared in PBS–diethanolamine buffer (1:1).

2.5 | Specificity of the assay

Cross-reactivity for AFB1-related compounds (AFB_2, AFG_1 and AFG_2) each prepared at concentrations of 10 ng/mL in PBS–diethanolamine buffer (1:1) were also tested.

2.6 | Real sample analysis

To 2.5 g of weighed food sample, 200 μL (10 ng/mL and 100 pg/mL) of known AFB_1 concentration was spiked and kept for 60 minutes at room temperature (RT, 27°C). To the above spiked sample, 5 mL of methanol was added and kept for shaking at RT for 30 minutes. After standing stratification, the supernatant was decanted in a clean 15-mL centrifuge tube, and this extraction process was repeated 2–3 times. The solvent fractions were pooled and centrifuged at 2000*g* for 5 minutes at RT to collect the residue-free supernatant. The supernatant obtained was then flash evaporated completely and dissolved in 1 mL dichloromethane to recover the sample. These samples were then kept in water bath at 40°C for dichloromethane to completely evaporate. The samples were then finally redissolved in 200 μL of 1:1 PBS–diethanolamine buffer in which further assays were carried out.

Standard deviation was calculated for the developed assay to determine the reliability of the method. The calibration curve for AFB_1 assay was prepared by plotting the concentration of AFB_1 on the *x*-axis against the relative fluorescence unit obtained on the *y*-axis.

3 | RESULTS AND DISCUSSION

Aflatoxins are a group of chemically similar toxic fungal metabolites (mycotoxins) produced by certain molds of the genus *Aspergillus* growing on a number of raw food commodities. Aflatoxins are highly toxic compounds and can cause both acute and chronic toxicity in humans and many other animals. The aflatoxins consist of about 20 similar compounds belonging to a group called the difuranocoumarins, but only 4 are naturally found in foods. These are aflatoxins B_1, B_2, G_1 and G_2. Aflatoxin B1 is the most commonly found in food and also the most toxic. Aflatoxins may be present in a wide range of food commodities, particularly cereals, oilseeds, spices and tree nuts. Maize, groundnuts (peanuts), pistachios, brazils, chilies, black pepper, dried fruit and figs are all known to be high risk foods for aflatoxin contamination, but these toxins have also been detected in many other commodities. Aflatoxins are quite stable compounds and survive relatively high temperatures with little degradation. Approximately 100 countries around the world have regulations governing aflatoxins in food and most include maximum permitted or recommended levels for specific commodities. The EU has set limits for AFB_1 and for total aflatoxins (B_1, B_2, G_1 and G_2) in nuts, dried fruits, cereals and spices.[3]

Limits vary according to the commodity but range from 2 to 12 μg/kg for AFB_1 and from 4 to 15 μg/kg for total aflatoxins.

3.1 | Competitive aptamer assay

The scheme for the competitive fluorescent aptamer assay developed in the present study is presented in Figure 1. The method was based on the principle of competitive binding of AFB_1 to AFB_1 hapten conjugate and aptamer specific to AFB_1 to obtain a concentration-based fluorescence enhancement. Optimization of the method was carried out by testing some key parameters. AFB_1 hapten-BSA conjugate was immobilized on to black 96-well immune wells, and AFB_1 was allowed to recognize and attach to the immobilized conjugate. The coating antigen used in the aptamer assay was AFB_1-BSA (100 ng/well), and in order to select the blocking reagent, 1% BSA, skimmed milk, casein and gelatin, commonly used as blocking reagents in typical immunoassays, were tested to avoid non-specific absorbance. The aptamer assay with 1% gelatin produced sufficient fluorescent signals with nil or negligent background fluorescence. Blocking for 2 hours gave very efficient fluorescent signals without any background signals as noise (data not shown). These optimized conditions were used for further studies.

Concentration of the AFB_1 aptamer (from 0.1 to 2μM) required for analyzing AFB_1 positive and negative sample was found to be 0.1μM (Figure 2). This concentration provided sufficient fluorescence for carrying out further studies. Results showed that the fluorescently labeled aptamer could recognize the attached AFB_1 and conjugate with it. The fluorescence of aptamer measured was found to increase with increase in AFB_1 concentration.

3.2 | Calibration curve

Free AFB_1 molecules are recognized by the conjugate (hapten-BSA). Optimal conditions for the aptamer assay for AFB_1 were determined,

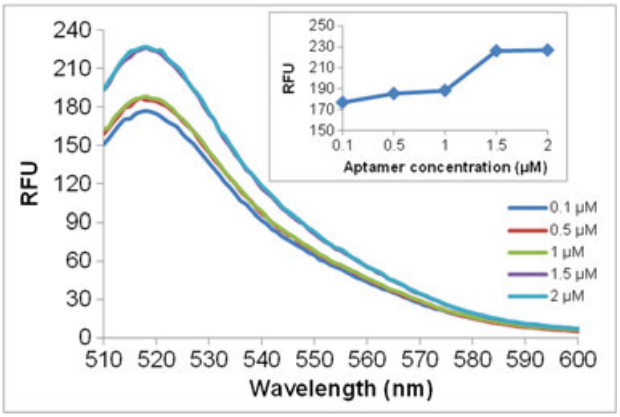

FIGURE 2 Optimization of aptamer concentration for AFB_1 aptasensor

and the detection limit was investigated using AFB_1 (standard) at concentrations of 10 pg, 50 pg, 100 pg, 10 ng, 25 ng, 50 ng and 100 ng, in 1:1 PBS–diethanolamine buffer. The lower detection limit for AFB_1 (5% inhibition) was found to be 10 pg (Figure 3), and below this concentration, there was no clear detectable fluorescence. Detection

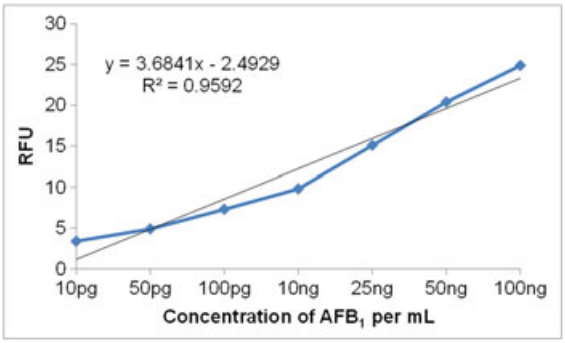

FIGURE 3 Fluorimetric detection of AFB_1 using aptamer assay

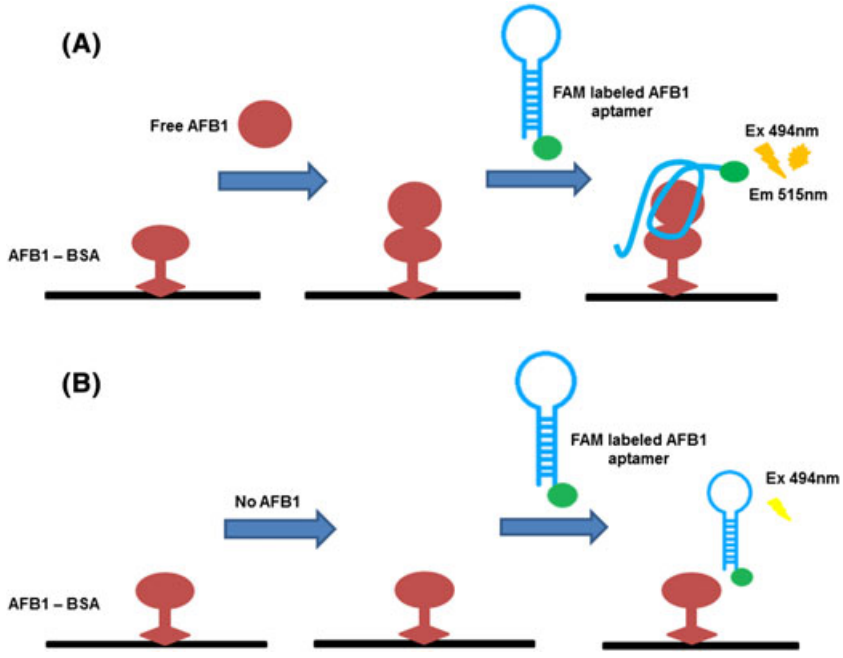

FIGURE 1 Scheme for the fluorescent competitive aptasensor for detection of aflatoxin B1 A, presence of target analyte B, absence of target analyte

could be done up to 100 ng after which the fluorescence was almost stable.

3.3 | Specificity of the assay

The specificity of the method and cross-reactivity to other aflatoxins was studied by carrying out the assay using AFB_2, AFG_1 and AFG_2, at a concentration of 10 ng/mL. The specificity of the aptamer was expressed in terms of percentage inhibition of activity. The AFB_1 aptamer showed maximum cross-reactivity with AFB_2 as compared to other toxins used for the study. This can be attributed be the extremely close structural similarity between the aflatoxin groups, which resulted in binding of the aptamer to AFB_2 (Figure 4).

3.4 | Precision and accuracy of the assay

The inter- and intra-assay precisions were determined at different AFB_1 concentrations viz. 10 pg and 100 ng levels. The intra-assay precision was assessed by analyzing 6 replicates of each sample in a single run, and inter-assay precision was assessed by analyzing the same by repeated analysis of samples of aflatoxin isomers (Table 1). The coefficient of variation of 5 assays of each standard was satisfactory, with less precision at lower analyte (AFB_1) concentrations.

3.5 | Analytical performance of the assay for AFB_1 detection in spiked food samples

In order to validate the feasibility of the fluorescent aptamer assay to detect AFB_1 in food samples, AFB_1-positive food samples were prepared by spiking AFB_1 at concentrations of 10 ng and 100 pg/g each. Since aflatoxins are major contaminants of spices like chili, pepper and groundnuts, these were used for real sample analysis. The results are shown in Table 2. Recovery of AFB_1 spiked in samples varied from 92% to 101% at 10 ng level and 92 to 102 at 100 pg level. There was no significant matrix interference from the raw materials studied except for chili, which showed some interference with marginally high recovery percentage. The results of the recovery study are in the acceptable range required by Directive 98/53/EC.[21]

The results of the recovery tests were also compared to that obtained using HPLC. Results show that the developed assay was more sensitive than the conventional technique of HPLC for the detection of AFB_1. Higher recoveries (124%–153%) at higher concentration (100 pg) and reasonably lower recovery of AFB_1 at lower concentration (10 ng, 52%–81%) were obtained using HPLC.

The results of the method based on the competitive aptamer-assay proposed in the present study with minimal matrix interference suggest that it can be extrapolated to other grains and food commodities as well. However, further studies would be required to validate the same.

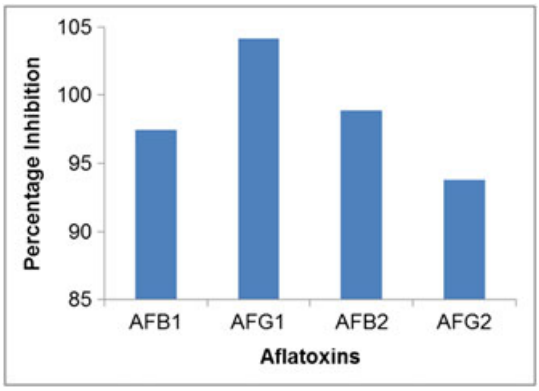

FIGURE 4 Percentage inhibition of different aflatoxins using AFB_1 aptasensor

TABLE 1 Inter- and intra-assay precision measurement of AFB_1 aptamer assay

AFB_1 concentration	Inter-assay (n = 6) (SD)	Intra-assay (n = 6) (SD)
100 ng	0.088	0.028
50 ng	0.054	0.045
25 pg	0.033	0.044
10 ng	0.048	0.032
100 pg	0.083	0.091
50 pg	0.034	0.026
10 pg	0.021	0.091

3.6 | Merits of the present method

This study describes a sensitive aptamer-based simple assay method that quantifies aflatoxin in food samples. Quantitative analysis of AFB_1 can be achieved by aptamer-based technique with high sensitivity (LOD of 10 pg in buffer system [PBS–diethanolamine buffer]). Linearity was observed in the range of 50 pg and 50 ng with a good regression coefficient (R^2) of 0.959.

Many chemical procedures have been developed to identify and measure aflatoxins in various commodities. The basic steps include extraction, lipid removal, cleanup, separation and quantification. The HPLC requires more cost per assay, sensitivity and specificity and also either pre- or post-derivatization for AFB_1 detection. Also, only 1 sample can be analyzed at a time which means that more time is required for analysis of a number of samples. Immunoassays, enzyme and aptamer-based sensors have also been described previously (Table 3). The use of antibodies raises several questions on the ethical use of animals for their generation. Also, batch-to-batch variations are expected which may alter the sensitivity of the developed method using antibodies. Although enzyme-based assays are sensitive and specific, they suffer from instability of enzymes with slight modification in physiological conditions. Aptamers can circumvent these problems and offer

TABLE 2 Comparative recovery studies using aptamer assay and HPLC

Food commodity	Spiked AFB_1 concentration	Aptamer assay recovery (%)	HPLC recovery (%)
Dried red chilies	10 ng	101.94	52.45
	100 pg	102.64	127.73
Pepper	10 ng	96.85	81.74
	100 pg	99.28	124.84
Groundnut	10 ng	92.51	72.51
	100 pg	92.87	153.98

TABLE 3 Various reported methods for detection of AFB_1

Method	LOD	Real sample analysis	Reference
Antibody-based direct competitive ELISA	0.1 μg/L	Rice	[19]
Spectrophotometric enzyme assay	10 ng/mL	Barley	[22]
Antibody-based indirect competitive ELISA	0.3 ng/mL	Popcorn, cornflakes, cornmeal, peanuts, pecans, peanut butter	[23]
Optical immunosensor	0.5 ng/mL	Barley, wheat	[24]
Chemiluminescence-based aptasensor	0.11 ng/mL	Corn	[25]
Quantum dots-graphene oxide	1 nM	Peanut oil	[14]
RT-qPCR aptasensor	25 fg/mL	Infant rice cereal, Chinese wild rye hay	[17]
Fluorescent competitive aptasensor	10 pg/mL	Dried red-chilies, groundnut, whole pepper	Present study

several advantages over techniques that use enzymes and antibodies in the format.

Moreover, synthesis of oligonucleotides is relatively easy and aptamers can be easy modified. They have been used in conjunction with proteins, fluorescent dyes or other tags for the detection of mycotoxins,[26] antibiotics[27] and other biomolecules.[25] Many of the studies for detection of AFB_1 using aptamers use the enzyme, horseradish peroxidase (HRP), as a marker in order to recognize the binding[24] or are based on immobilization of the aptamer to the immunoreagents or solid phase.[28] To the best of our knowledge, this is the first study on fluorescence-based competitive aptamer assay for AFB_1 using FAM. The main advantage of using fluorescent tag is that the result signal can be easily achieved rapidly. The FAM-AFB_1-aptamer complex in the developed assay produced sufficient fluorescent signals to allow discrimination between AFB_1 positive and negative samples.

Thus, the proposed technique is a suitable tool for the rapid screening of AFB_1 because of its high sensitivity, selectivity, simplicity, reliability and applicability. This technique can detect the target accurately and selectively, even in food samples without much food matrix interference and thus can be successfully used for the detection of AFB_1 in biological samples.

4 | CONCLUSIONS

In this study, we successfully developed a fluorescent-based aptamer assay for AFB_1 using a FAM-tagged aptamer and applied the method to real samples. To the best of our knowledge, a FAM-based competitive aptamer assay for the analysis of AFB_1 has not been reported previously. After optimizing the assay by testing key parameters, the sensitivity of the method was found to be appreciable to detect AFB_1 at low concentrations. The aptamer used in the study had extremely low cross-reactivity to other aflatoxins. The recovery values obtained in the present study using real samples met the requirements of the EU for newly developed analytical methods and demonstrated the feasibility of the aptamer assay for application on real samples. This study provides great opportunities to develop a biosensor platform for detection of AFB_1.

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