A NEW BIOSENSOR PLATFORM FOR THE DETECTION OF AFB1: REAL TIME ELECTROCHEMICAL PROFILING (REP™) ASSAY METHOD

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Abstract – we introduce a new biosensor platform (MiSens) for the detection of aflatoxin B1 (AFB1) based on real time electrochemical profiling (REP™) assay method coupled with the use of natural and artificial antibodies (polymers) as affinity ligands. Sample clean-up was performed with aflatoxin-targeted polymer and the samples were then analysed using natural antibody-based MiSens biosensor assay. The new biosensor allows real-time and on-site detection of AFB1 in foods with a rapid, sensitive, fully automated and miniaturized system and expected to have an immense economic impact for food industry.

Keywords: Aflatoxin, Real-time electrochemical profiling, MiSens biosensor, HPLC

1. INTRODUCTION

Mycotoxins are secondary metabolites mainly produced by filamentous fungi on various food and feedstuffs. Among mycotoxins, aflatoxins (AFs) are considered to be the most toxigenic fungal metobolites with carcinogenic, mutagenic, teratogenic and immunosuppresive effects. Aflatoxin is one of the most common mycotoxins in grains. The occurrence of AFs is influenced by certain environmental factors; hence, the extent of contamination varies with geographic location, agricultural and agronomic practices, and the susceptibility of commodities to fungal invasion during preharvest, storage, and/or processing periods [1, 2]. Four types of AFs including B1, B2, G1, and G2 commonly occur in the natural environment even though 20 different AFs have been identified [3]. Aflatoxin B1 was classified as Group 1 carcinogen (carcinogenic to humans) by the International Agency for Research on Cancer (IARC) [4]. Aflatoxins have been found in grains and herb medicines in humid conditions [2]. The permissible limits established by European Commission for AFB1 and total AFs are permitted 2 µg kg-1 and 4 µg kg-1, respectively in all cereals and products derived from cereals, including processed cereal products [5, 6].

Solid-phase extraction (SPE) has appeared as a simple alternative to be coupled with a variety of methods for aflatoxin detection to provide easy and cost effective techniques [7]. The applications of SPE can be widely seen also in imprinting technology as it offers simple and successful clean up procedures. Molecularly imprinted polymers (MIPs) have increasingly been introduced in many analytical procedures, mainly due to their high selectivity in comparison to other stationary phases. MIPs have attracted significant attention as substitutes for natural receptors in chromatography, sensors and assays [8, 9]. The reason for this is coming from the fact that molecular imprinting is arguably the most generic and cost effective technique for preparing synthetic receptors. Imprinted polymers have several important advantages as compared to natural receptor molecules: (i) polymers can be prepared for practically any compound; (ii) they have similar affinity to natural biomolecules but often have better specificity; (iii) polymers can work in organic solvents and they are stable at low/high pHs, pressure and temperature [7].

Most of the current methods for quantitative AFs determination include chromatographic methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and recently liquid chromatography tandem mass spectrometry (LC-MS/MS), suitable for use in regulatory laboratories [10]. Several immunology

based semi-quantitative and qualitative methods including enzyme linked immunosorbent assays (ELISAs) were also developed. However, these techniques require qualified personnel, time consuming sample pre-treatment and assay procedure and expensive laboratory equipments. These tests also entail sample transportation from storage to the laboratories that cause increased waiting time and high costs. The novel AFs detection systems for rapid screening and detection in field and laboratory applications are dip-stick kits [11], optical-based sensing methods (e.g. hyperspectral imaging and electronic noses) [12], and biosensors [13]. As The global food safety testing market by contaminants is estimated to grow at an annual growth rate of 10.46% to $2.5 billion in 2015, the need for easy and cost-effective testing solutions increase [14]. Here we introduce a new biosensor platform for the detection of AFB1 based on real time electrochemical profiling (REP™) assay method coupled with the use of natural and artificial antibodies (polymers) as affinity ligands. Sample clean-up was performed with aflatoxin-targeted polymer and the samples were then analysed using natural antibody-based MiSens biosensor assay.

The sensor has abilities such as low cost unique sensor chip, simple instrumentation, automatization, high sensitivity, rapidity and applicability to a wide range of analytes. It relies on the fundamental basics of electrochemical immunosensing, in particular amperometry. With a new electrode array and a novel microfluidic channel design, all the steps of the assay including the immobilization, binding and amperometric measurements have been performed during the fluid flow. Compared to the conventional immunoassay platforms such as ELISA that takes around 40 minutes, this technique provides automated and fast assay period in 25 minutes.

2. METHOD

2.1. Materials and reagents

Phosphate buffered saline (PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) tablets and AFB1 standard were purchased from R-Biopharm Rhone Ltd. (Glasgow, UK)., horse radish peroxidase (HRP), 3,3',5,5'-Tetramethylbenzidine (TMB) ready to use reagent (contains H_2O_2), hydrochloric acid (HCl), methanol (MeOH), N,N-dimethylformamide (DMF), ethylene glycol dimethacrylate (EGDMA) and N,N-methylenebisacrylamide (MBAA) were purchased from Sigma-Aldrich (Poole, UK). Protein A was purchased from Thermo Scientific (Istanbul, Turkey). AFL-HRP conjugate was obtained from Romer Labs (Tulln, Austria). Oxygen free argon was purchased from Habas (Istanbul, Turkey). Ultrapure water (18 MΩ cm^{-1}) was obtained from a Milli-Q water system (Millipore Corp., Tokyo, Japan).

2.2. Synthesis of NIPs and cartridge preparation

The polymers were manufactured according to the procedure reported by Piletska [15] with minor modifications and in the absence of template molecule. This novel non-imprinted polymer (NIP) designed for aflatoxin was prepared as following: N,N-methylenebisacrylamide (MBAA, 331.5 mg, 2.15 mmol) was dissolved in 3287 µl DMF, and mixed with EGDMA (6300.4 mg, 15.9 mmol) and 1,1-azo-bis(cyclohexane) (46.08 mg). After ultra-sonication for 5 min, the monomer mixture was purged with Argon for 7 min and sealed. It was polymerized by UV for 45 min and then was incubated at 75 °C in the oven 24 h for polymerization. The polymer monoliths were ground using a manual mortar and then sieved. Polymer fraction with size 63-125 µm was collected. The particles were washed using Soxhlet extraction.

2.3. MiSens detection assay

MiSens biosensor relies on the REP™ technology where all the steps of the assay including the immobilization, capturing and amperometric measurements are performed during the fluid flow. Towards an innovative miniaturized automated system, a novel electrode array has been designed and integrated to a microfluidic system. This new sensor chip consists of 6 working electrodes (1 mm diameter) with shared reference and counter electrodes, a sensor cassette made of poly(methyl methacrylate) (PMMA) was designed and a silicon o-ring has been used to create a microfluidic channel (~7 µl) on the electrode arrays.

2.4. Preparation of wheat sample

Artificially contaminated 50 g wheat sample was prepared by spiking of 0.1 ml and 0.02 ml AFB1 stock standard solution (1 µg ml-1) in benzol to obtain final concentrations 2 ng g-1 and 0.4 ng g-1 in wheat, respectively. It was allowed to dry

overnight at ambient temperature in the fume hood prior to extraction.

2.5. Extraction

50 g samples were mixed with distilled water (100 ml), MeOH (150 ml) and sodium chloride (5 g), and blended at high speed for 3 min. The slurry were transferred to 500 ml erlenmayer flask containing 250 ml distilled water and mixed with magnetic stirrer for 1 min. After mixing, the slurry was filtered through filter paper (Whatman 4). The crude extracts were filtered (syringe filter 0.44 µm, Millipore MF membrane) and later used for the biosensor detection assay.

2.6. Clean-up with solid phase extraction (SPE)

MBAA polymer particles (100 mg) were packed into 1 ml SPE cartridges with fritted polyethylene diskettes at the bottom and top and they were placed in a 12-vial vacuum manifold (Agilent VacElut 12, Agilent technologies) connected to a vacuum pump. SPE columns containing 100 mg of blank IA-based polymer were conditioned with 5 ml H2O and 1 ml of the filtered extract was then cleaned up on the SPE column at a flow rate of 0.5 ml min-1. The column was washed with 2 ml of MeOH: H2O (2:8) with a flow rate of 0.5 ml min-1. AFB1 was then eluted with 1 ml MeOH: Dichloromethane (1:1). The eluted extract was then analyzed by HPLC and electrochemical sensor.

2.7. Clean-up with immunoaffinity column (IAC)

10 ml of clear wheat filtrate was pipetted into reservoirs containing 40 phosphate buffered saline (PBS) solution and placed on immunoaffinity column (Aflaprep, R-Biopharm Rhone Ltd, Glasgow, UK). The solution was passed through the immunoaffinity column at a flow rate of around 3 ml min-1. Column was washed with 20 ml water and dried by applying little vacuum for 5–10 seconds or passing air through with a syringe for 10 seconds. Samples were eluted from the column by passing 1 ml of HPLC grade MeOH and then 1 ml of HPLC grade water by gravity to collect the eluate into a glass vial. 100 µl of the eluate was injected into the HPLC.

2.8. MiSens assay for aflatoxin detection

MiSens biosensor relies on the REP™ technology where all the steps of the assay including the immobilization of the capture protein, binding of the conjugate protein and amperometric measurements with substrate have been performed during the fluid flow. For all steps of the assay, flow rate and quantity are the key factors. The REP™ assay with respect to assay flow rate, reagent concentrations (protein A, anti-AFL and AFL-HRP conjugate) and reagent volume was optimized. At first, the AFB1 standard solution was used to determine the calibration curve.

Using the automated MiSens biosensor, the aflatoxin antibody (300 µl, 5 µg ml-1 in PBS buffer) was bound on protein A immobilized surface via 30 µl min-1 fluid flow. Later 100 µl aflatoxin samples/standards were mixed with 200 µl aflatoxin-HRP conjugate, and injected to sensor surface (30µlmin-1). The electrochemical measurements were applied at -0.1 V potential between reference and counter electrodes simultaneously with 50 µl min-1 fluid flow. The current measurements were taken during PBS buffer injection to obtain a baseline and then the subsequent injection of 240 µl 3,3',5,5'-Tetramethylbenzidine (TMB) (50 µl min-1) incurred a current change (due to the surface bound HRP). The distinctness between the current obtained during TMB and buffer was used as signal correspond to target concentration. By injecting 0.1 M HCl (150 µl, 100 µl min-1) twice, the interaction between the aflatoxin antibody and protein A was disrupted hence antibody is removed from the surface; allowing surface regeneration. Therefore, after each regeneration step, the sensor surface was ready to test new sample/standard concentration with fresh capture antibody. Three data points were used to obtain the mean and standard deviation of the results. The limit of detection (LOD) was calculated as the signal obtained from the assay that is equivalent to the 3 times the standard deviation of the signals obtained from the blank standards. An LOD of 2 ppb was obtained.

3. RESULTS

In this work AF standards and contaminated food matrix (wheat) were used and the developed NIP columns and the REP™ platform (MiSens biosensor) were compared with the reference methods. With this aim, MiSens system was utilized after extraction using NIP and the results were compared with IAC-HPLC.

NIP-SPE column exhibited high binding capacity

in the solvent (80% methanol). Full retention of the analyte was achieved after direct loading of the wheat extract. Washing the column with 2 ml PBS solution at pH 9.2 assured the analyte remaining on the column and removing some interference from the column. Complete elution was accomplished using 1 ml methanol and the eluent was analyzed both by HPLC and REP assay.

3.1. NIP performance tests with standard solutions

The performance of NIP-SPE column was evaluated by HPLC comparing to affinity column (IAC). To prove the SPE column extraction success, we measured AFB1 standard solution before and after the extractions in different concentrations. The recovery values were found to be 96%, 108% and 89% for 10 ppb, 20 ppb and 40 ppb concentrations, respectively.

3.2. NIP performance tests with matrix (wheat)

The polymers were packed into SPE columns and evaluated under similar chromatographic conditions. The column was washed with MeOH: H2O (2:8) with a flow rate of 0.5 ml min-1. AFB1 was then eluted with MeOH: Dichloromethane (1:1). The eluted extract was then analyzed by HPLC and electrochemical sensor. 0.4 ppb of AFB1 in spiked wheat samples was found as 0.35 ppb (87.5% recovery) and 0.33 ppb (82.5% recovery) for IAC-HPLC and NIP-HPLC, respectively. 2 ppb of AFB1 in spiked wheat samples was determined as 1.86 ppb (93% recovery), 1.73 ppb (86.5% recovery) and 1.88 ppb (94.0% recovery) for IAC-HPLC, NIP-HPLC and NIP-MiSens, respectively.

4. CONCLUSION

These results clearly indicate that the produced NIP column works as effective as immunoaffinity columns used in the study. In addition, the aflatoxin detection performance of the NIP coupled MiSens assay is comparable to immunoaffinity column coupled HPLC results; hence, can be utilized as an alternative on-site aflatoxin detection method.

As a conclusion; The new biosensor allows on-time detection of mycotoxins in real samples using NIPs and antibodies with a rapid, sensitive, fully automated and miniaturized system and will have an immense economic impact for food industry.

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