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Miniaturised hybrid immunoassay for high sensitivity analysis of aflatoxin M1 in milk☆

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ABSTRACT

An ultra-sensitive sandwich ELISA was developed for detection of AFM1 in milk. The assay involved the immobilization of rat monoclonal antibody of AFM1 in 384 microtiter plate to capture AFM1 antigen. This was detected by tracer secondary rabbit poly-clonal antibody labelled with horseradish peroxidase upon addition of a luminol-based substrate. Milk samples with different fat percentage were analyzed after pre-treatment. Linear range of AFM1 detection 250–6.25 pg/mL was achieved in 3% fat milk. The miniaturised assay (10 μL) enabled ultra trace analysis of AFM1 in milk with much improved lower limit of detection at 0.005 pg/mL. A sensitive magnetic nanoparticles (MNPs) based ELISA was also developed and coupled with micro plate ELISA for analysis in milk. The hybrid-assay, by coupling the 1°Ab immobilized MNPs column with microwell plate assay enabled simultaneous measurement of low (0.5 pg/mL) and high AFM1 contamination (200 pg/mL). The most promising feature of this MNPs-ELISA is the small column size, high capture efficiency and lower cost over other reported materials. The proposed assay can be deployed for simultaneous analysis and monitoring of AFM1 in milk.

1. Introduction

Aflatoxins are highly toxic mycotoxins produced by *Aspergillus* species in a wide range of food and animal feedstuffs stored under temperature and humidity conditions favorable to mold growth. When aflatoxin B1 contaminated feed is ingested by cattle, it is transformed into its hydroxylated product, AFM1, which is then secreted in the milk. AFM1 is known for its hepatotoxic and carcinogenic effects. The toxic and carcinogenic effects of AFM1 recently lead WHO-IARC to change its classification from group 2 to group 1 (IARC, vol. 82, 2002). AFM1 is relatively stable during milk pasteurization, storage as well as during the preparation of various dairy products (Codex Committee, 2001; Badea et al., 2004). To date, aflatoxins are regulated in many countries worldwide. Due to the fact that milk intake in infants is high and when young they are vulnerable to toxins, the European Community legislation imposes maximum permissible levels AFM1 of 50 ng/L in milk and 25 ng/L for infant formulae (Henry et al., 2001). To minimize the occurrence of AFM1, it is essential to trace the sources of contamination using rapid, selective, sensitive and cost effective assays. Several methods for AFM1 determination have been developed. High-performance liquid chromatography (HPLC) (AOAC Official Method, 2000.08), thin layer chromatography (TLC) (Kamkar, 2006) and enzyme-linked immunosorbent assays (ELISA) (Rastogi et al., 2004) are mainly used in routine analysis. For an effective screening and monitoring of AFM1 in foodstuffs such as milk at ultra low level, analytical methods combining simplicity with high detectability and analytical throughput are required. This can be achieved by means of immunological methods in conjunction with a highly sensitive detection of the label (Magliulo et al., 2005). HPLC, TLC techniques require extensive sample preparation steps and well-trained personnel. Moreover, the reagents and instrumentation used are expensive (Thirumala-Devi et al., 2002). Immunochemical techniques are becoming very popular for mycotoxins analysis with many literatures reporting the use of a commercially developed ELISA (Thirumala-Devi et al., 2002; Lopez et al., 2003; Rodriguez et al., 2003; Rastogi et al., 2004). ELISA is not only suitable tool for quick and sensitive analysis with high sample throughput, but also cost-effective and requires only a small sample volume for analysis (Pei et al., 2009; Parker and Tothill, 2009). Among the established ELISA techniques, sandwich-type immunoassay is an effective bioassay due to the high specificity and sensitivity (Knopp, 2006).

Enzyme labels have experienced widespread popularity since their first use in 1971 in an ELISA (Van Weeman and Schuurs, 1971). Enzyme labels are not consumed, and their reactions can

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be initiated and stopped. Furthermore, enzymes amplify the signal because an enzyme can produce many detectable molecules, up to 10^7 molecules of substrate per minute per enzyme molecule, by its catalysis of a substrate product reaction. They can be used in both homogeneous and heterogeneous immunoassays. Enzymes are the most commonly used labels as they can produce colored, fluorescent, luminescent, and electroactive compounds enabling detection by a variety of techniques (Gracia et al., 2005). Enzyme labels detected by chemiluminescent (CL) substrates, such as the luminol (5-aminophthalyhydrazide)/peroxide/enhancer system for horseradish peroxidase (HRP) or dioxetane-based substrates for alkaline phosphatase represent the most sensitive detection system in immunoassay development. CL compounds produce light in response to chemical reactions and as labels in immunoassay, they can be more sensitive than radio labelled and fluorescent forms (Krick and Wild, 2001). In addition, the CL signal detection can be performed immediately after substrate addition, thus shortening the overall analytical procedure when compared with conventional colorimetric assays (Magliulo et al., 2005). Gracia et al. (2005) have reported that luminol could be used as an enzyme substrate for HRP that yield high sensitivity.

Owing to their high surface area, nano-materials can facilitate miniaturization and thereby provide enhanced number of binding sites. MNPs have special relevance in bio-analytical chemistry because of their larger surface to volume ratio, enhanced antibody–antigen kinetics, lower mass transfer resistance and easy separation of immobilized bio-molecules from reaction mixture using magnetic field (Yu, 1998). Recently, widespread use of MNPs in biosensors has been witnessed through a flurry of literature (Cheng et al., 2005; Nikitin et al., 2007; Ravindranath et al., 2009). Various antibody coupling strategies using nanoparticles have also been reported (Wang et al., 2006; Radoi et al., 2008; Wang and Gan, 2009; Ahirwal and Mitra, 2010). The covalent binding of antibody through self assembled monolayer is more suitable than the other conventional methods like physical adsorption and polymer entrapment (Liu et al., 2006; Shankaran and Miura, 2007). The advantages of using covalent binding over physical adsorption to anchor antibodies and other proteins to a substrate surface are well documented in the literature (Ivanova et al., 2006). Despite MNPs prevalent applicability in biosensor field, reports on MNPs being deployed for analysis of AFM1 have been scarce. In one such testimony, reported by Radoi et al. (2008), AFM1 detection was done using MNPs coated with protein G by physical adsorption.

In the present work, we report a novel approach where a highly sensitive microplate sandwich ELISA was developed and integrated with MNPs which could detect ultra trace amount of AFM1 in milk. The functionalized MNPs were used as an affinity capture column wherein antibodies immobilized on their surface could capture AFM1 from milk sample. To circumvent interference attributed to whey proteins and fat, the effect of pretreatment of milk sample with trichloro acetic acid (TCA) followed by centrifugation and filtration was investigated to assess the performance of integrated ELISA. The miniaturised assay was used for high throughput analysis of AFM1 which showed an astoundingly low detection limit 0.005 pg/mL as against other reported ELISA (Thirumala-Devi et al., 2002; Rastogi et al., 2004; Magliulo et al., 2005). Moreover the assay was validated by performing recovery studies in certified reference material for AFM1 (ERM-BD 282).

The proposed assay further demonstrated the use of functionalized MNPs as affinity capture material through a miniaturised column for AFM1. This gave better efficiency of capture and cost effectiveness when compared to materials like protein A and protein G (Fuentes et al., 2005; Radoi et al., 2008) in affinity column.

2. Materials and methods

2.1. Materials and instrumentation

AFM1, bovine serum albumin (BSA), Tween 20, luminol, protein A agarose fast flow, 50% (v/v), protein G sepharose 4B fast flow (recombinant protein G, in 20% ethanol) and iron oxide (Fe_3O_4, average particle size 15 nm), certified reference material ERM-BD282 (AFM1 in whole milk powder, <0.02 μg/kg) were purchased from Sigma–Aldrich (USA). Hydrogen peroxide (H_2O_2) 30% (w/v), acetonitrile (ACN) HPLC grade, trichloro acetic acid (TCA), sodium chloride (NaCl), methanol (99% pure) were purchased from Merck (Germany). Anti AFM1 fractionated antiserum primary antibody (1°Ab) raised from rat and HRP conjugated secondary antibody (2°Ab) raised from rabbit were purchased from Abcam (UK). 3-Aminopropyl-triethoxysilane (APTES) 99% pure and glycerol (99% pure) were purchased from Acros Organics (USA). Glutaraldehyde (GA) solution (25%) was purchased from Merck (India). Sodium hypochlorite (4%) solution was purchased from Fisher Scientific (India). Sonication of the sample was done in Toshcon ultrasonic cleaner (Toshniwal process instruments Pvt. Ltd., India). Centrifugation, shaking and filtration of the samples were done by Spinwin mini centrifuge, Spinix shaker and syringe filter, purchased from Tarsons (India). 0.22 μ filter papers (25 mm diameter) were obtained from Millipore (USA). White 384 well polystyrene microtiter plates were purchased from Nunc (Denmark). HybridSPE^{TM} (30 mg/1 mL) SPE tubes were purchased from Supelco (USA). Flow analysis was done by a multi-channel pump, Gilson, Minipuls Evolution (France). For CL measurement, VictorX^4 2030 optiplate reader from Perkin Elmer (USA) was used. Glove box, Cole Parmer (USA) was used for the handling of AFM1 standard solution. Water produced in a Milli-Q system (Millipore, Beford, MA, USA) was used for preparing all the solutions. Certified ultra high pure nitrogen (99.9%), pH meter (Seven Multi Mettler Toledo, 8603, Switzerland) were used. Commercial milk samples of different fat content were purchased from the local supermarket of Goa, India.

2.2. Preparation of buffers

Buffers were made by the following method. For coating purpose, a 0.05 M carbonate buffer (CB) was prepared. The pH was adjusted to 9.6. As CB changes composition over time, it was made fresh each time. 0.01 M phosphate buffered saline (PBS) was used for incubation and washing purpose. The pH was adjusted to 7.4. Another washing buffer (PBST) was made by adding 0.05% Tween 20 (v/v) in PBS. All buffer solutions were stored at 4 °C when not in use. The blocking solution was prepared by adding 2.5% (w/v) BSA to PBS.

2.3. Preparation of AFM1 standard solutions

All the AFM1 solutions were prepared inside a Glove box in a maintained inert N_2 atmosphere. AFM1 stock solution was prepared by dissolving the AFM1 powder in 5% ACN (v/v) in PBS at a concentration of 5 μg/2 mL and stored at −20 °C. In order to develop the assay, meeting regulatory requirements of European Commission (50 ng/L), working standard solutions in the range of 250–0.005 pg/mL were prepared by diluting the stock with 5% ACN [safety note: aflatoxins are highly carcinogenic and should be handled with extreme care. Aflatoxin contaminated labware should be decontaminated with an aqueous solution of sodium hypochlorite (4%)].