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Enzyme immunoassay for monitoring aflatoxins in eggs

L. Anfossi*, F. Di Nardo, C. Giovannoli, C. Passini, C. Baggiani

Department of Chemistry, University of Turin, Via Giuria, 5, I-10125, Turin, Italy

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ABSTRACT

Rapid and sensitive competitive enzymatic immunoassays for measuring most relevant aflatoxins in eggs have been developed by synthesizing two hapten derivatives. Polyclonal antibodies raised against a hapten obtained from aflatoxin B1 (AFB1) were exploited to set an AFB1-selective assay, whereas antibodies obtained through immunising with a hapten derived from aflatoxin M1 (AFM1) allowed us to detect four principal aflatoxins (B1, G1, B2, and G2) and the most relevant AFB1 metabolite (AFM1) with detection limits in eggs of 0.3 μg kg^{-1} for AFB1, AFG1, and AFM1 and 3 μg kg^{-1} for AFB2 and AFG2, respectively. We also established a rapid and simple protocol for extracting aflatoxins from eggs by employing aqueous methanol (70%) followed by partitioning with hexane to remove fats. The whole analytical process is simple, very rapid (the extraction requires 14 min, and the assay is completed in 30 min) and proved to be accurate and precise enough (recoveries ranged from 84 to 100% and RSD% were within 20% for intra- and inter-assay experiments) to be proposed as a first level screening method for the monitoring of the occurrence of aflatoxins in egg.

1. Introduction

Aflatoxins are secondary metabolites produced by moulds of the *Aspergillus* family, which contaminate several crops, including cereals, oilseeds, tree nuts, and spices. Due to the fact that *Aspergillus* moulds could grow on crops pre-, during, and post-harvest and that their toxic metabolites are very stable to chemical and physical stresses, aflatoxins have been found in raw and processed materials and represent the most common cause of chemical contamination of foodstuffs, according to the European Union alert system (EU Rapid Alert System for Food and Feed). Among about 300 different natural aflatoxins, the most diffuse and toxic is the aflatoxin B1 (AFB1). It is produced by *Aspergillus flavus* and *Aspergillus parasiticus* and has been recognized as the most potent carcinogen for human (International Agency for Research on Cancer, 2002). Besides AFB1, principal aflatoxins are: aflatoxin B2 (AFB2), which is produced by the same mould as AFB1 but in a lesser extent; aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2), which belonged to *A. parasiticus* (AFG1 is the predominant toxin excreted by *A. parasiticus*). Maximum acceptable levels for AFB1 and for the sum of all the four aflatoxins have been set worldwide in commodities susceptible to contamination and intended for human consumption (European Commission, 2010) or for feeding farm animals (European Commission, 2003). Furthermore, it has been demonstrated that dairy cattle, sheep and goats fed with AFB1 contaminated feedstuffs transfer the aflatoxin to milk partially as the unmodified precursor, but primarily as a hydroxylated metabolic product (Van Egmond, 1989). This AFB1 metabolite excreted to milk (aflatoxin M1, AFM1) retains most of AFB1 toxicity (Caloni, Stammati, Friggé, & De Angelis, 2006) (International Agency for Research on Cancer, 2002); therefore, maximum tolerable levels have been established also for AFM1 in milk (European Commission, 2010). Conversely, the carry-over of AFB1 into meat of animals fed with contaminated material is controversial (Hayes, Polan, & Campbell, 1977) (Díaz-Zaragoza et al., 2014) (Hussain et al., 2010) and the risk for consumers associated to meat consumption seems to be negligible. Recently, the potential AFB1 carry-over into eggs in laying hens fed with contaminated crops has been investigated. Pandey and Chauhan reported on the effect of ingesting AFB1 contaminated grain on chicks (Chauhan, & Pandey, 2007). AFB1 residues were detected in eggs and breast muscle of AFB1-fed hens. The carry-over of AFB1 was confirmed by the works of Hassan et al. (Hassan, Khan, Khan, Javed, & Hussain, 2012) and of Herzallah (Herzallah, 2013) who also studied the combined effect of the four major aflatoxins. He found an analogous carry-over for AFB1 and the other three aflatoxins. Hassan et al. observed that AFB1 residues appeared in eggs after 5 days from starting administration of contaminated feedstuffs and

* Corresponding author. Tel.: +39 0116705219; fax: +39 0116705242.
E-mail address: laura.anfossi@unito.it (L. Anfossi).

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that AFB1 accumulated in eggs with increasing amounts found for protracted feeding with contaminated grain. Nevertheless, the amounts of aflatoxin residues found in eggs were very low in every case and varied between 0.01% (Herzallah, 2013) and 0.07% (Hassan et al., 2012) of the aflatoxin intake. This result could be partially explained by the fact that AFB1 is metabolized by the bird (Rawal, Kim, & Coulombe, 2010). Indeed, the metabolic transformation of AFB1 was responsible of diseases observed on hens and highlighted by the same authors. However, none of the preceding papers considered the metabolic detoxification pattern which led to the formation of hydroxylated metabolites of AFB1 (AFM1 and aflatoxin Q1) (Rawal et al., 2010) and authors did not investigate the occurrence of AFM1 in eggs, similarly to what is done in milk.

World egg production involved over 60 millions tonnes per year from a total of approximately 6.5 billion hens and expanded by more than two per cent a year in the last decades (Nutriad). China is the world largest egg producer and is accounted for one third of the entire world production, followed by USA and India. Countries belonging to the European Union produce approximately 7.5 million tonnes of egg per year (European Commission Agricultural and Rural Development). The demand of feed for sustain poultry production makes suspect on its quality, also because most of the ingredients used to produce poultry feed are used for human consumption. Thus, the risk that materials discarded for human consumption could be employed as feedstuffs is not negligible. Furthermore, since poultry production is relatively inexpensive and widely available and, as poultry meat and eggs are considered low-cost sources of protein, their production is strongly encouraged in developing countries, which led sometimes to not adequate housing and management of animals and feedstuffs and to increased risk of contamination (FAO, 2013).

Therefore, the accessibility of rapid, cost-effective, and simple methods of analysis to detect aflatoxins in eggs would help scientists to better investigate the occurrence of these contaminants and to more adequately support conclusions on risks for human health due to consumption of eggs belonging to hens fed with aflatoxin contaminated materials. Moreover, it would allow the efficient and continuous monitoring of such contaminants to assure food security. Analytical methods to determine aflatoxins in eggs currently available are based on chromatographic techniques coupled to fluorescence or mass spectrometric detection (Herzallah, 2009) (Garrido Frenich, Romero-González, Gómez-Pérez, & Martínez Vidal, 2011) (Capriotti, Cavaliere, Piovesana, Samperi, & Laganà, 2012). However, to ensure the rapid and cost-effective screening of large numbers of sample and the availability of analytical methods applicable in developing countries, the exploitation of immunochemical methods of analysis, which are known to address requirements of rapidity, simplicity and inexpensiveness, is advisable.

This study aimed at developing a rapid and sensitive competitive enzymatic immunoassay for measuring most relevant aflatoxins in eggs. Therefore, two hapten derivatives were synthesized, with the objective of raising polyclonal antibodies able to bind the principal aflatoxin (AFB1), the main AFB1 metabolic product (AFM1) and possibly the other three relevant aflatoxins (AFG1, AFB2, and AFG2). By exploiting those antibodies, two direct competitive immunoassays could be proposed: an AFB1-selective assay and a group-selective assay. This last allowed us to detect all above-mentioned mycotoxins. Moreover, aflatoxin extraction from eggs was optimized with the aim of fulfilling the same requirements of rapidity, easy operation and cost-effectiveness to allow the applicability of the whole analytical protocol as a screening method in routinary monitoring of aflatoxin contamination in eggs.

2. Materials and methods

2.1. Materials

Aflatoxin B1, aflatoxin M1, aflatoxin B2, aflatoxin G1, aflatoxin G2, ochratoxin A (OTA), deoxynivalenol (DON), fumonisin B1 (FB1), and zearalenone (ZEA) standard solutions were Oekanal certified solutions from Sigma Aldrich (St. Louis, MO, USA). Aflatoxin B1 and aflatoxin M1 powders were purchased from Fermentek (Jerusalem, Israel). Bovine serum albumin (BSA), N,N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), and 3,3′5,5'-tetramethylbenzidine liquid substrate (TMB) were purchased from Sigma- Aldrich (St. Louis, MO, USA). Horse-radish peroxidase (HRP) was purchased from Roche Diagnostics (Milan, Italy). Sephadex G-25 cartridges were from GE Healthcare (Milan, Italy). Dimethylformamide (DMF), methanol (HPLC grade) and all other chemicals and microtitre plates were obtained from VWR International (Milan, Italy).

2.2. Production of the hapten, hapten-protein conjugates and antibodies

Aflatoxin B1-O-(carboxymethyl)oxime (AFB1-cmo) and Aflatoxin M1-O-(carboxymethyl)oxime (AFM1-cmo), were synthesized from AFB1 and AFM1, respectively, as previously reported (Chu, Hsia, & Sun, 1977). The two haptens (Fig. 1) were conjugated to BSA by the DCC/NHS ester method and used for immunization; AFB1-cmo was also conjugated to HRP to generate the labelled probe. Briefly, equimolar amounts of AFB1-cmo or AFM1-cmo, DCC and NHS were dissolved in anhydrous DMF and the mixture was incubated at 4 °C temperature for 2 h. Proper amounts of the mixture were then added to protein solutions prepared in 0.13 M $NaHCO_3$, to obtain a final molar ratio of 200:1 (AFB1-cmo or AFM1-cmo:BSA), and 10:1 (AFB1-cmo:HRP). BSA conjugates (AFB1-BSA and AFM1-BSA) were incubated overnight at room temperature, while the HRP conjugate (AFB1-HRP) was reacted for 1 h at room temperature. Separation of conjugates from by-products and excess of reagents was carried out by gel filtration on a Sephadex G-25 cartridge (mobile phase: phosphate buffer saline).

Anti-AFB1 and anti-AFM1 antibodies were produced by Davids Biotechnologie (Germany) by using their standard immunization protocol for rabbit polyclonal antibodies (Davids Biotechnologie) and sera were collected after 70 days from the first injection. The immunoglobulin fraction was obtained from antisera by ammonium sulphate precipitation and used without further purification.

2.3. Competitive direct ELISA

We prepared the immunoreactive solid phase by coating wells with 150 μl of anti-AFB1 or anti-AFM1 rabbit polyclonal antibodies diluted in carbonate/bicarbonate buffer pH 9.6 (overnight at 4 °C). To assure complete saturation of well surface, after washing plates with 0.05% Tween 20, we further incubated 300 μl of phosphate buffer supplied with 0.15 M NaCl and 0.5% BSA (PBS@BSA) for 1 h at room temperature, followed by washing wells with 0.05% Tween 20.

The construction of calibration curves involved mixing 100 μl of AFB1-HRP (0.08 μg ml^{-1}) in PBST@BSA and 100 μl of AFB1 standards diluted in aqueous methanol (35%) at concentrations ranging from 0 to 2500 μg l^{-1}. After 15 min incubation in immunoreactive wells, unbound reagents were removed by five washings with a washing solution including 0.3 M NaCl and 0.05% Tween 20. Colour development was obtained by a 15 min incubation with TMB (200 μl per well). The addition of 50 μl of sulphuric acid (2 M) stopped colour development and allowed absorbance recording at 450 nm. For egg samples, extracts prepared as described below were directly added to wells instead of AFB1 standards. All