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Application of ELISA for cost-effective analysis of aflatoxins in foods and feeds

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Abstract

Agricultural commodities are often vulnerable to attack by fungi that are able to produce toxic metabolites called mycotoxins. Among the various mycotoxins, aflatoxins have assumed significance due to their deleterious effects on humans, poultry and livestock and their role in carcinogenesis and immunosuppression. Simple and cost-effective methods for determining aflatoxin levels in various commodities are extremely important. In addition to such methods being used by commercial companies, research institutes and farmers, they are indispensable during risk assessment analyses and for determining the suitability of agricultural commodities for international trade. This paper describes the application of various forms of enzyme-linked immunosorbent assay (ELISA), and their merits for the estimation of aflatoxins in foods and feeds. A cost-effective penicillinase-based ELISA is described and the reliability of ELISA techniques, as compared with other methods of evaluating aflatoxins, are outlined.

Key words: aflatoxins; mycotoxins; antibodies; ELISA; immunoassay.

1. Introduction

Mycotoxins are fungal secondary metabolites that are toxic to humans and livestock. The majority of economically important mycotoxins such as aflatoxins, ochratoxins, cyclopiazonic acid, fumonisins, trichothecenes and zearalenone are produced by *Aspergillus*, *Penicillium* and *Fusarium* species. According to the Food and Agriculture Organization of the United Nations (FAO), over 25% of agricultural commodities world-wide are significantly contaminated by mycotoxins (Boutrif 1995). Among mycotoxins, aflatoxins are the most important since they are hepatocarcinogenic, immunotoxic, and can lead to growth retardation in children (Turner *et al*. 2003). They comprise a group of more than 15 aflatoxins, with B1, B2, G1 and G2 often being found in agricultural commodities. Aflatoxin B1 is by far the most toxic as well as ubiquitous of all the aflatoxins. Consumption of aflatoxin B1-contaminated feed by cattle leads to excretion of aflatoxin M1 into their milk. Since large quantities of milk are consumed, permissible levels of aflatoxin M1 are lower than those for aflatoxin B1 (<0.05 mg/kg or 0.05 mg/l). It is important to be able to detect and quantify aflatoxins in foods and feeds so that humans and animals can be protected from their harmful effects. Due to stringent regulations imposed by developed countries, only aflatoxin-free agricultural commodities are suitable for international trade.

Analytical techniques available for quantifying aflatoxins include those based on biological,

physicochemical and immunological procedures. Biological methods for measuring aflatoxins are based on the measurement of their toxic effects on animals, which include death and pathological lesions; however, these analyses are not very specific and their sensitivity is low. Physicochemical methods for analysing aflatoxins, such as thin layer chromatography, high performance liquid chromatography and gas chromatography-mass spectrometry, are laborious; they involve expensive instrumentation and clean-up of the samples, and are thus of limited use. Immunochemical methods of aflatoxin analysis, however, offer several advantages over biological and physicochemical methods (Chu 1986). They are cost-effective, sensitive, specific, provide rapid results and are less expensive to perform than the majority of analytical methods. This article describes recent developments in the application of immunological techniques such as enzyme-linked immunosorbent assays (ELISAs) for estimation of aflatoxins in foods and feeds.

ELISAs are by far the most widely applied immunological techniques and are based on the recognition of an antigen or a hapten by antibodies. The result of any binding reaction between an antibody and an antigen is made visible by means of an enzymatic marker. The two major requirements for the application of ELISAs are high quality antibodies and methodologies to use the antibodies for the quantification of aflatoxins.

2. Production of polyclonal antibodies

Antibodies are serum glycoproteins belonging to the immunoglobulin class and are produced by the vertebrate immune system against high molecular mass foreign material; polyclonal antibodies contain a mixture of antibodies to various epitopes. Since aflatoxins have a low molecular mass (330 Da), they are not immunogenic and have to be coupled to a carrier molecule, usually a protein, in order to induce an antibody response in the vertebrate immune system. The sensitivity and the selectivity of the resulting antibody can depend on the location of the coupling site to the carrier, the way in which coupling is undertaken, and the number of aflatoxin molecules that bind to one carrier molecule. The protein carriers commonly used are bovine serum albumin (BSA), ovalbumin and keyhole limpet haemocyanin (KLH). Among them, KLH is regarded as a superior carrier because it is foreign to the vertebrate immune system.

Aflatoxins conjugated to BSA are often used for the production of antibodies in rabbits or other animals. Multiple-site immunization at weekly intervals followed by a booster (usually five or six injections) often does not yield high titre antibodies. A modification is to use several subcutaneous injections (often >7) at multiple sites followed by a non-immunization rest period of 6 to 8 months. Booster subcutaneous injections after this rest period results in the production of high titre antibodies (Thirumala-Devi *et al.* 1999, 2002a).

3. Preparation of samples for ELISA

Samples (e.g. peanuts, cereals, poultry feeds) are thoroughly mixed, and ground to a fine powder in a Waring blender. For each assay, a sub sample of 15 g is extracted in 75 ml of a mixture of methanol-water and KCl (70:30:0.5%) by blending in a Waring blender followed by shaking for 30 min. The extract is filtered through Whatman No. 41 filter paper.

For processing by ELISA, samples are diluted to give two-fold to ten-fold step-wise dilutions in phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and 0.2% BSA (PBS-T/BSA). Samples which are not known to contain aflatoxins should be included as controls.

Milk samples (usually 15 ml) are centrifuged at ambient temperature, and an equal volume of methanol is added followed by shaking for 30 min. The extract is filtered through Whatman No. 41 filter paper. For samples of powdered milk [including certified reference material (CRM)] and milk-based confectionery, a 10 g sample is suspended in 100 ml distilled water, heated to 50°C, homogenized in a Waring blender, and then processed in a similar manner to liquid milk. Methanol extracted and filtered test samples are diluted to 1:10 in PBS-T/BSA.

The above procedures have been applied widely for the estimation of aflatoxins in cereals (Ramakrishna *et al.* 1990), maize (Abbas *et al.* 2002), and poultry feeds (Thirumala-Devi *et al.* 2002b). Recently, it has also been applied for the estimation of AFM1 in milk and milk based products (Sibanda *et al.* 1999; Thirumala-Devi *et al.* 2002a; Rodriguez *et al.* 2003). When the aflatoxin concentration is below the ELISA detection limit, it is essential to carry out further cleanup using aflatoxin affinity columns. If resources are not

available to buy the columns, aflatoxins can be concentrated prior to ELISA treatment by a simple procedure involving addition of distilled water and chloroform. 1 ml methanol extract, 0.5 ml distilled water and 1 ml chloroform are mixed in a separating funnel and shaken vigorously, after which the lower chloroform layer is collected and evaporated to near dryness in a water bath at 60°C.

4. Choice of plate ELISA

ELISA can be performed in 96 well polystyrene plates or on nitrocellulose or nylon membranes. Assays in plates can often produce good results, while those performed on membranes give less consistent data.

4.1. Indirect competitive plate ELISA (IC-ELISA)

ELISA plates (high affinity) are coated with aflatoxin-BSA conjugate at 100 ng/ml in 0.2M sodium carbonate buffer, pH 9.6 (150 ml/well) and incubated overnight in a refrigerator. This is followed by blocking with 0.2% BSA prepared in PBS-T/BSA. In the third step, diluted polyclonal antiserum (at a constant dilution, often higher than 1:80 000) prepared in PBS-T/BSA in 50 ml aliquots is added to 100 ml of various dilutions of sample or toxin standards ranging from 100 ng/ml to 100 pg/ml. The amount of antibody bound to the toxin is estimated by the addition of goat anti-rabbit or goat anti-mouse immunoglobulins conjugated to an enzyme and the addition of a suitable substrate.

4.2. Direct competitive plate ELISA (DC-ELISA)

ELISA plates are coated with 100 ml (per well) of polyclonal antibodies at a dilution exceeding 1:40 000. Blocking is performed with 200 ml PBS-T/BSA. Toxin standards or test samples prepared in 7% MeOH/PBS at 50 ml volume, together with 50 ml of toxin labelled with an enzyme (diluted in PBS-T/BSA) are added to each well. Toxins present in the sample compete with the labelled toxin for binding to the antibody coated onto the well surface. The enzyme-labelled toxin attached to the antibody is then detected by adding a suitable substrate.

4.3. Choice of enzyme labels and conjugation

Enzyme labels widely used in ELISAs include alkaline phosphatase (ALP) and horseradish peroxidase (HRP). More recently, penicillinase (PNC) has also been employed. ALP and PNC are conjugated to commercially produced aflatoxin-BSA using the single bridge glutaraldehyde method (Clark and Adams 1977), while the periodate oxidation method is used for conjugation of aflatoxin-BSA with HRP (Tsang *et al.* 1995). The substrate used for ALP is *p*-nitrophenyl phosphate (1 mg/ml), while 3,5,3′,5′ tetramethylbenzidine (TMB)/peroxidase is used for HRP.

Although the reaction kinetics of ALP are linear, the enzyme and its substrate are expensive and are not readily available in developing countries. The reaction kinetics for HRP are not linear and its substrates are not readily available, especially in developing countries. A DC-ELISA with PNC-labelled aflatoxins has recently been developed. PNC activity is measured as the pH-mediated colour change of bromothymol blue, caused by the release of penicilloic acid. The sensitivity of detection of aflatoxins using PNC as a marker enzyme is comparable to that when ALP is used. Cost of the analysis using PNC is less than half of that with ALP.

4.4. Estimation of toxin concentration

A regression curve is drawn with the help of computer software, plotting $\log_{10}$ values for aflatoxin standards on the X-axis and optical densities at A_{405}, A_{450} and A_{620} for ALP, HRP and PNC systems, respectively, on the Y-axis. Aflatoxin concentrations ranging from 12.5 ng to 390 pg produce a linear curve; for the majority of estimations, this range is adequate. If the aflatoxin levels fall below 390 pg then the test will not give accurate results (Thirumala-Devi *et al.* 2002a; Reddy *et al.* 2001). If such samples require analysis, it may be necessary to concentrate the sample (see Section 3). Toxin concentration per gram is calculated using the formula:

$$AFB_1\ (\mu g/kg): \quad \frac{A \times D \times E}{G} \quad \text{or} \quad \frac{A \times E}{C \times G}$$

where:

A = AFB_1 concentration in diluted or concentrated sample extract (ng/ml)

D = Degree of dilution with buffer

C = Degree of concentration after cleanup

E = Extraction solvent volume used (ml)

G = Sample weight (g)

5. Membrane-based ELISA

The basic principle of membrane-based ELISAs is similar to that of DC-ELISA. Appropriate dilutions of polyclonal or monoclonal antibodies (at a volume of 3 ml in PBS) are first added to immunodyne immunoaffinity nitrocellulose membranes (pore size 0.45 mm). Since these membranes have a high affinity for proteins, it is essential to block the free protein-binding sites (not covered by the antibodies) of the membranes; this step is usually performed with 2% milk powder prepared in PBS-T/BSA. The enzyme-labelled toxin is then mixed with either aflatoxin-standards of known concentrations or with the sample in PBS containing 7% methanol. Samples not containing toxin are prepared accordingly to serve as a negative control. If toxin is present in the sample, it will compete with the labelled toxin for binding to the antibody. The enzyme-labelled toxin attached to the antibody is then detected by adding an insoluble chromogenic substrate. Such a substrate for HRP is TMB membrane peroxidase, while Sigma Fast TM Fast Red TR/Naphthol is used for ALP.

Several modifications to the basic membrane-based ELISA procedure are widely used. Membranes may be spotted with rabbit antimouse IgG before blocking is carried out. Anti-aflatoxin monoclonal antibodies may also be added before the addition of either toxin standards or test samples, followed by addition of a toxin-enzyme conjugate. Any enzyme that is retained is detected by suitable insoluble substrates.

After attachment of either primary or secondary antibodies and blocking with milk powder, membranes may be housed in a flow-through apparatus (Sibanda *et al.* 1999), which facilitates membrane handling. An inexpensive plastic tray can also be used if such an apparatus is not available.

The colour intensity of the spots produced by ELISA can be visually compared to that of toxin free samples (negative control). Control samples would show the most intense colour, whereas samples containing the lowest toxin concentration also show the most intense colour. The toxin concentration that fails to produce any colour is considered to be the visual detection limit.

There are several advantages of utilizing membranes over plates for performing ELISA. These include their facility to detect aflatoxins in extremely small volumes of test material, their suitability for testing samples in the presence of minimal laboratory facilities, and their ability to give very quick semi-quantitative results.

6. Costs of analysis

Comparisons of efficiency and cost-effectiveness for analysing peanut samples by DC-ELISA, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) are presented in Table 1.

7. Reliability of ELISA

In much of the literature, comparison of ELISAs with HPLC and liquid chromatography/mass

spectrometric techniques (Thirumala Devi *et al.* 2002b; Reddy *et al*. 2001; Abbas *et al.* 2002) produces comparable results, after allowing for permissible error limits. However, in one study, results using ELISA were considered to give increased variation in replicates than when HPLC was employed (Nilufer and Boyacioglu 2002), although the study concerned used a commercial kit. Due to the high costs involved, however, the test was not optimized to give consistent results.

In direct ELISAs using the PNC enzyme, results from 532 peanut samples obtained from farmer's fields were statistically analysed. The minimum detectable concentration for aflatoxin B1 was 1.4 mg/ml, with 0.05 probabilities of false positives and false negatives.

8. Conclusion

It is apparent that ELISA is reliable for quantitative estimation of aflatoxins in various foods and feeds. It is cost-effective and can be performed in developing countries in laboratories which have minimum facilities, while extensive cleanup which is often required for analytical techniques is not necessary. It should be mentioned, however, that in samples containing interfering substances, the procedure can give false positives.. It is important to include samples that have been shown by a different analytical method, such as HPLC, not to contain toxins. The ability to estimate aflatoxins is vital so as to eliminate or minimize the aflatoxin content. ELISA has great potential for analysis of a large number of samples within a short time and at a low cost. Therefore it serves as a valuable tool for risk assessment to human and animal health. In addition, ELISA is very versatile and possibilities exist for further improvement.

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Table 1. Comparisons of efficiency and cost-effectiveness for analysing peanut samples by direct competitive-ELISA, thin layer chromatography (TLC) and high performance liquid chromatography (HPLC)

Sample details	TLC	HPLC	ELISA by reagents produced in-house*	ELISA by commercially available kits
Number of samples handled for extraction per day	8	4	60	60
Number of samples analysed per day	36	24	160	160
Cost per sample ($)	1.9	21	0.90	12
Ability to analyse more than one sample at a time	Not very efficient	Possible	Possible	Possible
Capacity of throughput	Not possible	Possible but difficult	Possible	Possible

*Cost by ALP will be $2.55

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$$AFB_1\ (\mu g/kg):\quad \frac{A \times D \times E}{G} \quad \text{or} \quad \frac{A \times E}{C \times G}$$

where:

A = AFB_1 concentration in diluted or concentrated sample extract (ng/ml)

D = Degree of dilution with buffer

C = Degree of concentration after cleanup

E = Extraction solvent volume used (ml)

G = Sample weight (g)