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Research paper

Immunofiltration assay for aflatoxin B_1 based on the separation of pre-immune complexes

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ABSTRACT

A new approach for quantitative determination of AFB_1 based on the separation of pre-immune complexes in the same immunoassay system has been developed. No additional step for the separation of pre-immune complexes is required. The method uses a test device for separation of pre-immune complexes from the free AFB_1-enzyme conjugate by filtration through the membrane strips spotted with anti-AFB_1 antibody. The bound enzyme conjugate was visualized by super-catalyzed reporter deposition (Super-CARD) signal amplification method. The measured signal intensity is directly proportional to the amount of AFB_1 present in the sample. The detection limit obtained by the present method was 15 pg/ml. The data on the analytical parameters indicate that the new format of AFB_1 detection in foodstuffs is reproducible, accurate and specific. The method is user friendly and does not require any costly equipment or a well-equipped laboratory.

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1. Introduction

Membrane-based immunoassays are now widely used for the detection of small molecules like mycotoxins, pesticides, drugs etc. (Pal and Dhar, 2004; Paepens et al., 2004; Wang et al., 2005). The most favored format presently used for their measurement is based on competitive reaction between an unlabeled analyte and a labeled analyte in the presence of limiting amounts of anti-analyte antibody. In these assays, the measured signal intensity decreases with increasing analyte concentration. On the other hand, macromolecules such as proteins having more than one antigenic determinant are measured by noncompetitive immunoassay. The signals obtained are directly related to the amount of the analyte present in the sample. These assays have the potential for improved sensitivity and working range compared with corresponding competitive assays. However, these types of assays are not applicable to small molecules because their low molecular masses preclude simultaneous binding of two antibody molecules. During the last decade, several alternative strategies have been reported for small molecules, but they are limited to microtiter plate technology (Acharya and Dhar, 2008; Anfossi et al., 2004; Giraudi et al., 1999).

We recently reported a cost-effective analytical device for performing an immunofiltration-based immunoassay without using a pump (Pal and Dhar, 2004). It has also been successfully applied for rapid estimation of mycotoxins by immunoassay (Pal and Dhar, 2004; Saha et al., 2007), homogeneous spotting of antibody over the membrane surface (Saha et al., 2006), the estimation of protein (Acharya et al., 2008) and for improved catalyzed reporter deposition (Super-CARD) method of signal amplification (Saha et al., 2007). The device is simple in construction and consists of two layers: nitrocellulose membrane strips and a rectangular piece of filter paper placed over a semirigid polyethylene card. The membrane strips were placed

Abbreviations: AFB_1, aflatoxin B_1; AFB_1-HRP, AFB_1-horseradish peroxidase conjugate; B-T, biotinylated tyramide; *p*-OH-PPA-casein, 3-(*p*-hydroxyphenyl) propionic acid-casein conjugate; BSA, bovine serum albumin; Super-CARD, Super-catalyzed reporter deposition method; 4CN, 4-chloro-1-naphthol; DAB, 3,3′-diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; Tween 20, polyoxyethylenesorbitan monolaurate.

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in intimate contact over the moist filter paper, forming an aqueous network of capillary channels between them. Because the void volume of the moist filter paper was sufficient, the solution applied over the membrane surface is uniformly absorbed without lateral spreading.

In this study, we have used the same device for carrying out a novel immunofiltration assay for the determination of AFB_1. The principle of the method is based on separation of pre-immune complexes (formed between anti-AFB_1 antibody and AFB_1-enzyme conjugate or sample) from the free AFB_1-enzyme conjugate by filtration through the membrane strips spotted with anti-AFB_1 antibody. The selectively bound AFB_1-enzyme conjugate was visualized by super-CARD amplification method (Fig. 1). In the absence of AFB_1, the complex passes through the spotted immobilized anti-AFB_1 antibody, whereas with increasing AFB_1 concentration, the free AFB_1-enzyme conjugate binds to the antibody. The measured signal intensity is linearly correlated to the concentration of the AFB_1 in the sample. The performances of the method and its applicability for the detection of AFB_1 in wheat and corn samples have been demonstrated.

2. Materials and methods

2.1. Materials and chemicals

Nitrocellulose membrane, pore size of 0.45 μm was from Millipore Corporation, Bedford, USA. Filter paper (No. 3) was from Whatman International Ltd. Maidstone, England. Polyclonal antibody was raised in New Zealand white rabbits using AFB_1-*O*-carboxymethyloxime-BSA conjugate as immunogen (Bhattacharya et al., 1999). The antibody has already been characterized by ELISA and was found to be highly specific for AFB_1. AFB_1-HRP conjugate, B-T and *p*-OH-PPA-casein were prepared according to the method described previously (Pal and Dhar, 2004; Saha et al., 2007). Most of the reagents and chemicals were obtained from Sigma, St. Louis, USA. For the present work, this antibody was purified by repeated precipitation with ammonium sulfate (50% saturation) followed by dialysis against phosphate buffered saline. It was passed through a BSA-Sepharose column to remove anti-BSA antibodies. The amount of protein estimated in the antiserum was 16 mg/ml (BCA). AFB_1 stock solution (0.25 mg/ml in acetonitrile) was stored in −20 °C. Working standards for conventional assay (0, 125, 250, 500, 1000, 2500, 5000 and 10,000 pg/ml) and ultrasensitive assay (0, 15, 30, 60, 125, 250, 500 and 1000 pg/ml) were prepared by diluting with assay buffer. The color intensities of the sample spots were determined by densitometry with an Image Scanner (Amersham Pharmacia Biotech) using the Magic Scan software (version 4.5) for image scanning and the Image Master Total lab software (version 1.11) for quantitation (Pal and Dhar, 2004).

2.2. Preparation of membrane strips

2.2.1. Ultrasensitive assay

The method used here was similar to the one described previously (Saha et al., 2007). Briefly, a rectangular piece of

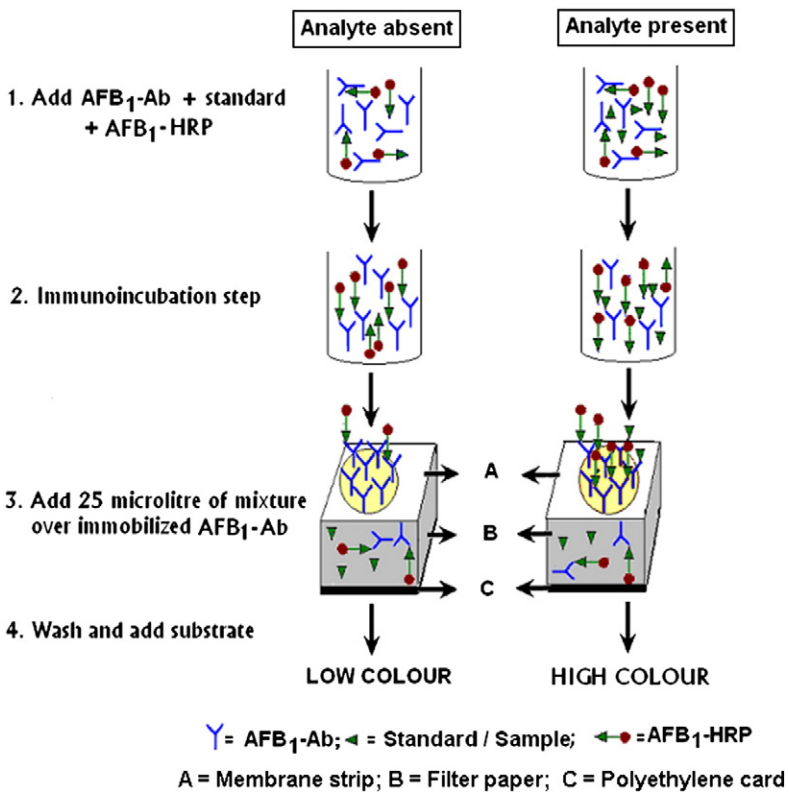

Fig. 1. Principle of immunofiltration assay based on separation of pre-immune complexes in the same immunoassay system.

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