Enzyme-Linked Immunosorbent Assay (ELISA) for Aflatoxin B_1 Estimation in Groundnuts

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Abstract

The commercially available hapten, afla B_1-oxime-bovine serum albumin, was used to produce an antiserum in rabbits. The same hapten was coupled with alkaline phosphatase (hapten-BSA-ALP) and used in the competitive direct enzyme-linked immunosorbent assay (ELISA) for the detection of aflatoxin B_1. Aflatoxin B_1 was extracted in methanol from naturally contaminated or 'spiked' groundnut seed samples.

Wells of a polystyrene microtitre plate were coated with the antiserum, the plates were washed in PBS-Tween, aflatoxin B_1 standards or groundnut sample extracts, and hapten-BSA-ALP conjugate were added and the plates incubated. The plates were again washed, and the amount of conjugate bound to the antibody was determined after addition of the substrate, p-nitrophenylphosphate.

The hapten-BSA-ALP conjugate has advantages in stability, simplicity of preparation, and high specificity, over the conventional toxin-enzyme conjugate in direct competitive ELISA. The assay method is more rapid and less expensive than the physico-chemical methods of aflatoxin analysis and it can detect levels of aflatoxin B_1 as low as 50 picograms.

Résumé

Détermination de l'aflatoxine B_1 dans les arachides par la méthode ELISA : *Le sérum albumine afla B_1-oximebovine, commercialisé sous le nom de Hapten, a servi à l'élaboration d'un antisérum chez des lapins. Ce produit est associé à la phosphatase alkaline enzymatique (Hapten-ALP) et utilisé dans l'essai ELISA adéquat pour la détection de l'aflatoxine B_1. Cette mycotoxine a été extraite avec le méthanol à partir des échantillons de graines d'arachide ayant subi une contamination naturelle ou celles artificiellement marquées.*

Les parois d'une plaque de microtitrage en polystyrène sont enduites d'antisérum et les plaques sont ensuite lavées au produit PBS-Tween; l'aflatoxine B_1 étalon ou les extraits des échantillons d'arachide ainsi que le conjugué Hapten-ALP y sont ajoutés avant incubation. Les plaques sont à nouveau lavées et la quantité de conjugué liée à l'anticorps est déterminée en y ajoutant le substrat p-nitrophénylphosphate.

Les avantages de Hapten-ALP par rapport aux conjugués toxine-enzyme classiques pour le

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test ELISA sont sa stabilité, sa simplicité et sa forte spécificité. La méthode est plus rapide et moins coûteuse que les procédés physico-chimiques de dosage des aflatoxines et possède un seuil de détection de l'aflatoxine B_1 inférieur à 50 picograms.

Resúmene

Determinación de la aflatoxina B_1 en el cacahuate por el método ELISA : *Se utilizó albúmina de suero bovino-afla B_1-oxime, conocido comercialmente con el nombre de "Hapten", para la producción de un antisuero en conejos. Este mismo "Hapten" fue ligado con fosfatosa alcalina (Hapten-BSA-ALP) y usado en el método directamente competitivo inmunoabsorbente vinculado a enzimas de cuantificación de anticuerpos, ELISA, para la detección de aflatoxina B_1. La aflatoxina B_1 fue extraída con metanol de muestras de semilla de cacahuate contaminadas, ya sea en forma natural o artificialmente, con esa micotoxina.*

Las depresiones de una placa de polistireno empleada para microtitulaciones se recubrieron con una capa delgada del antisuero; las placas se lavaron con el producto PBS-Tween; se agregaron soluciones calibradas de aflatoxinas B_1 o extractos de las muestras de cacahuates y, a continuación, el conjugado Hapten-BSA-ALP; luego se incubaron las placas. Se lavaron nuevamente las placas, y la cantidad de conjugado ligado al anticuerpo, se determinó después de la adición del substrato, p-nitrofenilfosfato.

El conjugado Hapten-BSA-ALP tiene marcadas ventajas de estabilidad, simplicidad y alta especificidad, sobre el uso del conjugado toxina-enzima convencional, en el método competitivo directo ELISA. El método analítico primeramente citado es más rápido y menos costoso que los métodos físico-químicos de análisis de aflatoxinas y puede detectar niveles de aflatoxina B_1 hasta del orden de 50 picograms.

Introduction

The thin layer chromatography (TLC) systems developed in the 1960s and 1970s are still the most commonly used methods for detection and estimation of aflatoxins in groundnut and several other agricultural commodities. These methods are expensive and time consuming and so efforts have been made to develop more rapid and less expensive methods for aflatoxin analysis (Pestka et al. 1981, Morgan et al. 1986, and El-Nakib et al. 1981).

Several enzyme-linked immunosorbent assay (ELISA) procedures have been reported for the estimation of aflatoxin B_1 in groundnut and groundnut products (El-Nakib et al. 1981, Fan and Chu 1984, Morgan et al. 1986). These assays have advantages over conventional analytical procedures using TLC and high pressure liquid chromotography (HPLC) in terms of speed, ease of sample preparation and use, and are potentially cheaper for aflatoxin analysis. The major application of ELISA procedures at present is analysis of aflatoxin B_1 in such agricultural commodities as maize, groundnut, and groundnut products (El-Nakib et al. 1981, Fan and Chu 1984, Morgan et al. 1986). A few direct and indirect ELISA procedures have been developed for analysis of aflatoxin B_1 in groundnut, groundnut meal, and peanut butter. All direct competitive ELISA procedures necessitate the use of aflatoxin-horse radish peroxidase (HRP) conjugate. Two problems are encountered in preparation of the conjugate: (1) instability of the toxin-

enzyme conjugate, and (2) variations in the amounts of toxin conjugated to the enzyme (Fan and Chu 1984). Both these factors affect the sensitivity of ELISA. To overcome these factors, we have used alkaline phosphatase (ALP) enzyme in place of HRP and have coupled it directly with the commercially available afla B_1-oxime-BSA. Using this afla B_1-oxime-BSA-ALP conjugate, we have developed a simple, rapid, specific, and comparatively inexpensive direct competitive ELISA for analysis of aflatoxin B_1 in groundnuts.

Materials and Methods

Chemicals and reagents

Aflatoxin B_1, bovine serum albumin (BSA, RIA grade), afla B_1-oxime-BSA, alkaline phosphatase (Type VII-NT), p-nitrophenyl phosphate disodium, glutaraldehyde (RIA grade), and Tween 20 ® were purchased from Sigma Chemical Co., St. Louis, MO, USA. Complete and incomplete Freund's adjuvants were obtained from Difco Laboratories, Detroit, MI, USA. Styrene microtitre plates were obtained from Dynatech Lab, Virginia, USA. All other organic solvents and inorganic chemicals used were of the highest analytical grade.

Production of antiserum against aflatoxin B_1

Antiserum against aflatoxin B_1 was produced by immunizing rabbits with afla B_1-oxime-BSA, using the methods of Chu and Ueno (1977) and El-Nakib et al. (1981). Antiserum titre was determined by the indirect competitive ELISA procedure described by Morgan et al. (1986).

Preparation of afla B_1-oxime-BSA-ALP conjugate

Afla B_1-oxime-BSA was conjugated to ALP through the glutaraldehyde bridge using the method of Avrameas et al. (1978). Several ratios of ALP and afla B_1-oxime-BSA were tried in initial experiments and the most suitable ratio was 1 mg of afla B_1-oxime-BSA to 4 mg of ALP. Afla B_1-oxime-BSA was dissolved in phosphate buffered saline (PBS, pH 7.4, 1 mg mL^{-1}) and mixed in proportions of 1:2, 1:4, 1:6, and 1:8 with ALP. The mixtures were dialyzed with 0.06% glutaraldehyde in PBS at room temperature for 2 h for conjugation. Excess glutaraldehyde was removed by dialyzing in PBS at 4°C for 3 h with three changes of PBS. The resultant afla -oxime-BSA-ALP conjugate was stored at 4°C.

Sample preparation and extraction

Groundnut seeds (cv J 11) were obtained from the 1986 rainy-season crop grown at ICRISAT Center. Healthy, mature, finely ground seeds (500 g) were divided into several lots of 5- and 10-g samples and these were 'spiked' with aflatoxin B_1 standard to give concentrations of 10, 20, 40, and 50 μg kg^{-1}. Samples were spiked by directly adding a measured volume of aflatoxin B_1 standard solution in methanol and then mixing thoroughly. These samples were used to test the recovery of the toxin by the ELISA procedure as described below. Some nonspiked samples were used to determine naturally occurring aflatoxin B_1. Naturally contaminated seeds were also tested for aflatoxin B_1 levels using TLC and ELISA procedures. For ELISA, aflatoxin B_1 was

extracted from the spiked or naturally contaminated samples with 55% methanol (5 mL g^{-1}) in a Waring blender for 3 min. The extract was filtered through Whatman No.1 paper, the filtrate concentrated by flash drying, and then diluted in PBS to avoid methanol interference (to have below 11% methanol in each assay). The Pons' method of extraction (Pons et al. 1966) was used in the detection and estimation of aflatoxin B_1 by TLC.

Aflatoxin B_1 standard

Various concentrations of standard aflatoxin B_1 ranging from 100 ng to 50 pg (in two-fold serial dilutions) in PBS containing 11% methanol were used in ELISA.

Direct Competitive ELISA Procedure

In the first step, 200 μL of crude antiserum (1:5000 in 0.05 M carbonate buffer, pH 9.6) were incubated in each well of polystyrene microtitre plates for 2 h at 37°C. The plates were then washed three times in PBS-Tween. Next, 100 μL of various dilutions of aflatoxin B_1 standard or sample extracts were added to each well, followed by 100 μL of afla B_1-oxime-BSA-ALP conjugate (diluted in PBS-Tween containing 1% BSA, 1:4000). The plates were incubated 37°C for 2 h. The wells were then washed with PBS-Tween, 200 μL of p-nitrophenyl phosphate (enzyme specific substrate) added, and the plates incubated at room temperature for 30 min. The reaction was stopped by the addition of 50 μL of 3 mol sodium hydroxide, and the absorbance at 405 nm was measured using a micro-ELISA reader.

Results and Discussion

The relationship between different concentrations of standard aflatoxin B_1 and absorbance values in the ELISA procedure is shown in Figure 1. All points are means of two replicates. There was no strictly linear relationship across the different concentrations of aflatoxin B_1 tested. This is probably due to reactions between different proportions of toxin and toxin-BSA-ALP conjugate that influence the amount of the toxin bound to the antibody adsorbed to the well surface. Only at optimal concentrations can a linear relationship be expected. A linear relationship can be obtained experimentally by utilizing a particular range of concentrations of the toxin and using a predetermined toxin-BSA-ALP conjugate dilution. The linear regression line (standard curve of aflatoxin B_1) is shown in Figure 2. This linear regression equation was used to calculate the toxin concentrations from the sample extracts.

Recovery of Aflatoxin B_1 from Spiked Groundnut Samples

The recovery of the toxin was 62–86% for 5-g samples and 70–107% for 10-g samples (Table 1). The recovery of the toxin was significantly lower when higher levels (50 μg kg^{-1}) of the toxin added than when lower levels (10 or 20 μg kg^{-1}) were added in the case of 5-g samples, while the recovery increased from 70 to 107% when 10-g samples were used. Similar results have been reported by other workers, particularly while using larger samples (Fremy and Chu 1984, Fan and Chu 1984, and El-Nakib et al. 1981). Dilution of the sample extracts would have avoided this problem. However, in both sample sizes, recovery of the toxin ranged from 70 to 96% from samples 'spiked' with concentrations of the toxin at 10 or 20 μg kg^{-1}. These results are supported