SENSITIVE ENZYME-LINKED IMMUNOSORBENT ASSAY FOR SCREENING AFLATOXIN B_1 IN FOOD-AND FEEDSTUFFS

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ABSTRACT

Aflatoxins are toxic metabolites produced by strains of *Aspergillus flavus* and *Aspergillus parasiticus*. They occur worldwide and contaminate certain foods and feedstuffs, especially wheat, corn, soya and groundnut products. Although the climate in Hungary is not favourable for production of these moulds, by consumption of imported foods these mycotoxins can cause serious health problems. As aflatoxin B_1 is highly carcinogenic, its accepted level is regulated in many countries, the official limit in Hungary is 5 ng/g. Five years ago our institute started a research program for developing ELISA tests to detect mycotoxins in food and feeds, resulted in reagent-kits for T-2, zearalenone and ochratoxin-A. This program continued by development of sensitive, monoclonal antibody-based direct, competitive ELISA test and reagent kit for quantitative measurement of aflatoxin B_1 with detection limit of 1.5 ng/g in cereals. The mean within-assay and interassay coefficients of variation of the standard curves were less than 10 %. With acetonitrile based extraction solvent the mean recovery values from cereals contaminated artificially with this mycotoxin was about 80 %. This test was offered for screening of samples in control laboratories.

MATERIALS AND METHODS

Monoclonal antibodies

Six-week-old female Balb/c mice were immunized subcutaneously (s.c.) with 100 µg of aflatoxin M_1-BSA emulsified with equal volumes of Freund's complete adjuvant. After one month the animal received intraperitoneal (i.p.) boosts of the same amounts of immunogens as given earlier, with Freund's incomplete adjuvant. Final immunizations were administered intravenously 3 d before fusion. Mice, having high titer of specific antibody against the immunogen were selected for further work. The fusion procedure was done according to Oi and Herzenberg (2). The supernatants of hybrid cells were tested with direct, competitive ELISA using aflatoxin B_1-peroxidase conjugate.

Peroxidase-conjugate

Aflatoxin B_1 was first converted to aflatoxin B_1-oxim by the method of Chu et al. (3) and then conjugated to peroxidase by active ester method according to Kitagawa et al. (4). The working dilution was determined by serial dilution of the conjugate.

Direct-competitive ELISA

The hybridoma supernatants were screened using direct, competitive ELISA, as described earlier by Barna-Vetró et. al. (5) for selection of monoclonal antibodies against ochratoxin A.

Recovery of Aflatoxin B_1 from artificially infected cereals.

Pure aflatoxin B_1 (1.5-9.0 ng/g) was added to 5 g of finely ground cereals (mixed feed, maize, soya) and the mixtures were homogenized 1 day prior to extraction. Samples were extracted with 15 mL of acetonitrile- 0.5% KCl - 6 % sulphuric acid (89+10+1) and agitated for 2 h at RT (about 22 °C) on a horizontal shaker. The extracts were let to sediment for 10 min. and the supernatants diluted to 1:10 with PBS-Tween 20 (0.1%) and used for assay in ELISA.

Calculation

Standard curve of aflatoxin B_1 was obtained by plotting $\log_{10}$ concentration (x-axis) against B/B_0 (y-axis). Toxin concentration of toxin in sample extracts were caculated by this formula.

B/B_0= (OD of standard or sample)/(OD of blank[no toxin added]), where optical density (OD) is the mean A 450 nm. The concentration of aflatoxin B_1 in sample extracts was calculated using the calibration curve and expressed in ng/g, after multiplication the ng/mL value by 30.

RESULTS AND DISCUSSION

Hybridoma production resulted in 10 stable clones with different crossreactions to related aflatoxins and with different 50% displacement values of B/B_0 for aflatoxin M_1 and aflatoxin B_1. The aim of our work was partly: to select monoclonal antibodies specific to aflatoxin M_1. This work supported by the EC (project No: CIPA-CT-93-0138) was used for the development of second generation immuno-assays (dipstick technology) for the detection of mycotoxin contamination in foods and feeds (6) - Second goal was to find aflatoxin B_1 specific monoclonal antibodies, which may be used for a sensitive ELISA test. Among the 10 clones four showed high reactivity for aflatoxin B_1, one of them (6G4F7/F3) was chosen for this work. The crossreaction with M_1, M_2, B_2 was 79, 33 and 76%, respectively. No crossreaction was measured with G_2, G_{2a} and B_{2a}. Using an isotype-specific ELISA the antibody was determined to be IgG_{2a} and λ. Direct competitive ELISA experiments were performed on ascitic fluid of 6G4F7/F3 clone. During the optimalization procedure the working dilution of the antibody, the peroxidase labelled conjugate, the reaction volume, time and temperature was determined. The detection limit (0 ± 2 SD) in buffer solution was 28 pg/mL. The 50 % displacement value (I_{50}) was 100 pg/mL aflatoxin B_1. The slope of the standard curve at the inflection point was 0.91. The within-assay and interassay coefficients of variation for standard concentration of aflatoxin B_1 (0.05 - 0.5 ng/mL) were <10%. The correlation coefficient (r) of the linear part of the calibration curve was 0.89, estimated by the Statistical Graphics program. Determination of the matrix effect is one of the important part of this developing work. For extraction of aflatoxin from cereals acetonitrile based solvent was used, which proved to be effective in other tests e.g. for T-2 and F-2 (7, 8). Recovery values of aflatoxin B_1 from artificially infected cereals averaged 80% as summarized in Table 1. The range of the test was 1.5 -15 ng/g with a detection limit of 0.9 ng/g. According to our preliminary experiences the aflatoxin B_1 ELISA is a useful tool for screening of samples in routine laboratories.

Table 1. Recovery of aflatoxin B_1 from artificially contaminated cereals

Added aflatoxin B_1 (ng/g)	Soya (ng/mL)	Soya detected %[b]	Maize (ng/mL)	Maize detected %[b]	Mixed feed (ng/mL)	Mixed feed detected %[b]
1.5	0.96 ± 0.085	64 ± 5.6	1.14 ± 0.19	761 ± 2.6	0.97 ± 0.15	65 ± 10
3.0	2.13 ± 0.32	71 ± 10.6	2.92 ± 0.22	97 ± 7.3	2.2 ± 0.22	73 ± 7.3
6.0	5.2 ± 0.7	86 ± 11.6	5.78 ± 0.27	97 ± 4.5	5.2 ± 0.49	87 ± 8.1
9.0	7.81 ± 0.91	87 ± 10.1	7.6 ± 0.52	84 ± 5.7	8.6 ± 0.95	96 ± 10.5

a Each sample was spiked in three parallel experiments. Values are means ± SD.
b Detected afla B_1 (ng/g)/added afla B_1 (ng/g) x 100

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