영남지방 농산물에 대한 위생학적 연구
(제 2보) ELISA 법에 의한 Aflatoxin B1 검색

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Hygienic Studies on the Agricultural Products in Youngnam Districts
(Part II) Determination of Aflatoxin B1 by ELISA Method

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ABSTRACT—A rapid, simple method of ELISA was applied for the determination of aflatoxin B1 in cereals from Youngnam districts. Antibodies obtained cross reacted with aflatoxin B2 and to a less extent with other aflatoxin B1 analogs. Response range for a typical standard curve was between 1 and 100 ppb. Fewer interference by spiked methanol-PBS-dimethylformamide extracts of rice was evidenced. Contents of aflatoxin B1 from rice (65) and barley (116) were determined by competitive direct enzyme- linked immunosorbent assay as follows. Three out of 65 rices samples were positive. Rice samples of R-18, R-30, and R-59 represent the aflatoxin B1 levels of 7.5 μg/kg, 3.5 μg/kg, 3.3 μg/kg, respectively, and showed 4.6% aflatoxin B1 contamination in rice samples. Meanwhile, four out of 116 barley samples were positive. UB-37 showed the highest aflatoxin B1 levels of 9.6 μg/kg and UB-35, UB-15 and UB-54 represent 7.5 μg.kg, 6.0 μg/kg and 3.6 μg/kg, respectively, and showed 3.4% aflatoxin B1 contamination in barley samples.

Keyword□Enzyme-Linked Immunosorbent Assay, Antibody, Cross reactivity, Aflatoxin B1.

Substantial economic losses of foods and feeds occur yearly due to deterioation by molds. In addition, growth of toxic molds in agricultural commodities are a potential threat to both human and animal health. Aflatoxins are known cacinogens, and the factors affecting the growth of aflatoxigenic molds and aflatoxin production in food products[1, 2], have received much attention. Conventional analytical methods for detection of mycotoxins that have been developed over the past 25 years typically employ biological assay, thin layer chromatography[3], high performance liquid chromatography[4, 5], gas chromatography, or mass spectroscopy[6]. But this requires highly skilled operators and elaborate equipment available only in special centres. The main limitation of all these methods in that samples require an extensive clean up procedure prior to analysis, adding to the time and cost of analysis[7, 8]. An alternative

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to such physio-chemical methods is biospecific analysis, employing antibody-mediated assay[9, 10]. These can be sensitive and specific, usually require little effort in terms of sample preparation, and are quantitative[11, 12]. The format employed in this laboratory as being the most suitable at present for the food industry is that of the enzyme-linked immunosorbent assay (ELISA) performed on microtiteration plates. On the other hand, in our country, there was no study about ELISA method for determination of any mycotoxins. In this report, we attempted to develop a competitive direct ELISA method for aflatoxin B1 and analyzed it from many cereal grains in Youngnam districts by ELISA method after only a single extraction with methanol phosphate buffered-saline demethylformamide.

MATERIALS AND METHODS

Samples—Cereals for the determination of aflatoxin B1 were obtained from 8 areas in Youngnam districts during march to July, 1989. 181 samples (rice: 65, barley: 116) were collected without fixed principle from local markets, home, or store house as could be seen at Table 1. As a post paper reported, some samples were deteriorated or mouldy. Levels of aflatoxin contamination were determined for each sample by ELISA.

Reagents—All inorganic chemicals and organic solvents were reagent grade or better. Bovine serum albumin (fraction V, fatty acid free) (BSA), chicken egg albumin (ovalbumin, grade III), 2,2-azinobis 3-ethylbenzthiazoline-6-sulfonate (ABTS), hydrogen peroxide, horseradish peroxidase, N,N-dimethylformamide, N,N-dicyclohexylcarbodiimide, N-hydroxysuccinimide, Tween 20, and all aflatoxin standards were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxymethoxylamine and dimethylformamide were purchased from Aldrich Chemical Co. Complete and incomplete Freund's adjuvant were purchased from Difco Laboratories. Rabbits (New Zealand white does) were purchased for immunization.

Table 1. Location of sampling site and source of samples in Youngnam districts.

Source	Total	Location
Rice	65	A(7), B(8), C(8), D(7), E(7), F(7), G(7), H(14)
Barley	56	A(8), B(7), C(7), D(7), E(7), F(7), G(7), H(6)
Unhulled barley	60	A(8), B(7), C(7), D(7), E(7), F(8), G(8), H(8)
Total	181	

A: Sangju, B: Porhang, C: Chinju, D: Kimhae, E: Milyang, F: Ulsan, G: Hamyang, H: Masan

Preparation of protein conjugates—Aflatoxin B1 was converted to aflatoxin B1-oxime as described by Roscoe *et al.*[13] Aflatoxin B1 oxime was conjugated to bovine serum albumine for use as immunogen and to horseradish peroxidase for use as enzyme marker by the N-hydroxysuccinimide procedure of Kitagawa *et al.*[14] Conjugates were kept at −20°C in 0.5 mg aliquots (0.5mg/m*l*).

Antisera preparation—Antiserum specific for aflatoxin B1 was produced against aflatoxin B1 oxime bovine serum albumin cojugates in rabbits as described by Chu *et al.*[8] Resulting serum was purified by ammonium sulfate precipitation and reconstituted to the original volume with 0.1 M sodium phosphate buffer (PBS, pH 7.5). Antisera were dialyzed against the same buffer for 48 h at 4°C and titered by ELISA as described below. The reactive specificity of the antibody obtained toward aflatoxin B1 and analogues was examined. Analogues used were aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2 and aflatoxin M1.

Preparation of sample extracts—Samples were ground into paste by coffee grinder (type Mcoc) and stand for overnight in dry oven (50°C). For analysis, 4g samples of each farm-product were weighed by Digital-gram, then transfer sample paste into flask (250 m*l*). Add 29 m*l* of 70% MeOH into flask, and blend for 20 min at high speed in a shaker. Ground samples were spiked for recovery experiments by adding aflatoxin B1 dissolved in methanol, mixing well and allowing the solvent to evaporate prior to extraction.

Direct competive ELISA—Direct competitive ELISA of the sample filtrates was described as follows: antiserum (1:500) was dried onto wells of

Blend at a 1:5 ratio for 5 minutes with extracting solvents
↓ filter
Mix filtrate with mycotoxin peroxidase conjugated
↓
Incubation in antibody coated microtiter well for 10 minutes
↓
Incubate substrate for 5 minutes
↓
Compare to positive and negative controls

Fig. 1. Steps for simplified microtiter well ELISA of mycotoxins.

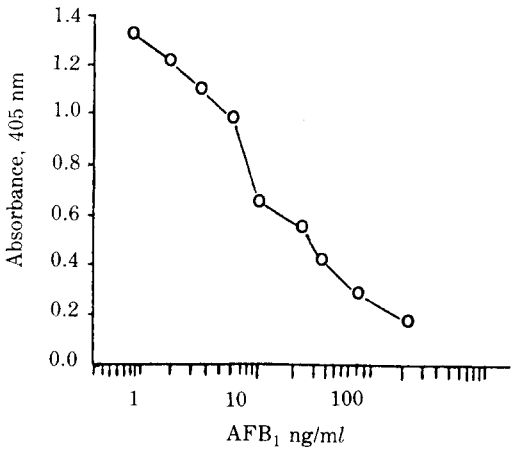

Fig. 2. Competitive direct ELISA standard curve for aflatoxin B1.

Table 2. Reactivity of aflatoxin B1 analogues in competitive direct ELISA.

Analogue	Cross-reactivity (%)
Aflatoxin B1	100
Aflatoxin B2	132
Aflatoxin G1	31
Aflatoxin G2	1
Aflatoxin M1	1

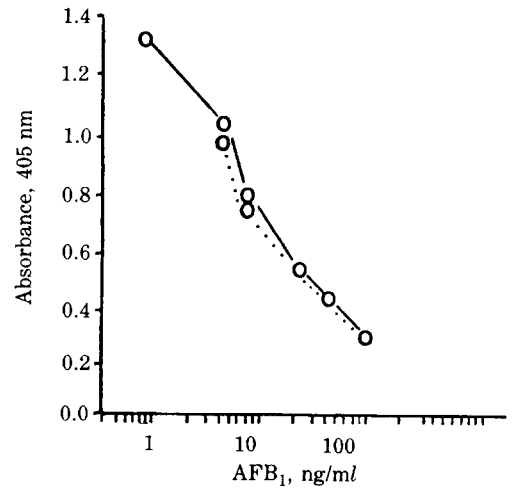

Fig. 3. Competitive direct ELISA standard curve for aflatoxin B1 in extractant and rice extracts.
O—O : Extraction solvent
O---O : Aflatoxin B1-free rice extract

a high blinding capacity NUNC microtiter plate (Vangard International Nepture, NJ) under forced air (40°C). Each well was eashed three times with 300 m*l* of washing buffer 0.01 M phosphate-buffered saline solution (PBS, pH 7.2) in 0.2% Tween 20 (PBS-tween). Aflatoxin B1 standard (diluted in 1:5 dilution of aflatoxin B1 free cereals prepared as described above) or sample extract (prepared as above without further dilution) were mixed in equal volume with aflatoxin B1-HRP (peroxidase cojugate) previously diluted 200-fold in blocking solution containing 1% dimethylformamide (vol/vol). 100 μl of the mixture was added to wells and the plate incubated for 10 min at 37°C. After the wells were washed 6 times as above, 100 μl of 2,2-azino-di-3-ethylbenthiazoline sulfonic acid (ABTS), substrate solution was terminated by the addition of stopping reagent (0.1% sodium aside in 0.3 M citric acid) and the absorbance at 405 nm was determined on the ELISA reader. Steps for simplified microtiter well ELISA of mycotoxins are described at Fig. 1, and standard curve was prepared shown as Fig. 2.

RESULTS AND DISCUSSION

Cross-reactivity—Results for the cross reactivity of different aflatoxin B1 analogues with the antibody in direct ELISA were shown in Table 2. Only aflatoxin B_1 cross reacted with the antibody and

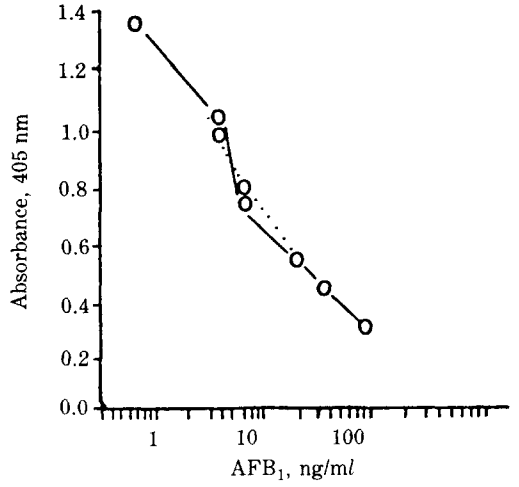

Fig. 4. Competitive direct ELISA standard curve or aflatoxin B1 in extractant and rice extracts.
O—O : Extraction solvent
O---O : Aflatoxin B1-free barley extract

Table 3. Content of aflatoxin B1 in rice analyzed by ELISA

No. of sample	Location	Aflatoxin B1 (μg/kg)
R-18	Ulsan	7.5±1.0
R-30	Hamyang	3.6±0.6
R-59	Chungmu	3.3±0.75

to a less extent with other aflatoxins. In gneral mycotoxin antibodies were extremely specific for the parent toxin and closely related structural analogues but did not show reactivity for unrelated chemicals found in agricultural commodities[15].

The effect of extract on ELISA—Before the determination of aflatoxin B1 from sample, standard curve was prepared shown as Fig. 2. Response range for a typical direct competitive ELISA standard curve was between 1 and 100 ppb. Potential interference in the ELISA by the sample extract was tested by comparing the standard curve prepared above and prepared in spiked methanol-PBS-dimethylformaniide extracts of samples. Partial interference by the sample extract could be estimated as might be predicted from Fig. 3 and Fig. 4. Further investigation of potential extract effects and alternative solvent extractants might

Table 4. Content of aflatoxin B1 in barley analyzed by ELISA

Sample	No. of sample	Location	Aflatoxin B1 (μg/kg)
Hulled barley (*56)	–	–	–
Unhulled barley (*60)	UB-15	Ulsan	6.0±1.2
	UB-35	Chinju	7.5±0.9
	UB-37	Chinju	9.6±1.2
	UB-54	Masan	3.6±0.5

*No. of sample analyzed

Table 5. Summary of natural occurance of aflatoxin B1 in rice and barley analyzed.

	Rice	Barley
Total	65	116
Positive	3	4
% of contamination	4.6	3.4

improve accuracy of the ELISA and suggested that ELISA and suitable for rapid semiquantitative screening of mycotoxin in agricultural products.

Determination of aflatoxin B1 in cereals—Quantitative analysis of aflatoxin B1 content of rice and barley was conducted with ELISA method. As could be seen in Table 3, R-18, R-30 and R-59 were positive for ELISA Test, recording that R-18, R-30 and R-59 were expressed aflatoxin levels of 7.5 μg/kg, 3.6 μg/kg, 3.3 μg/kg and 75 μg/kg, respectively. That is to say, three positive samples contained aflatoxin B1 at levels less than 10 μg/kg. We also have surveyed 116 barley samples by ELISA and found that only contained detectable aflatoxin B1. As a consequence of direct competitive ELISA, 56 hulled barley samples were negative for aflatoxin B1, that is, hulled barlrey were free from aflatoxin B1. Meanwhile, in the unhulled barley, four from 60 samples were positive for aflatixin B1 by ELISA, revealed that ranging between 3.6 μg/kg and 9.6 μg/kg. In case of the unhulled barley, all of which were below the 10 μg/kg action level as could be seen Table 4.

ELISA have been demonstrated to be specific, rapid, and sensitive alternatives to conventional chemical methods currently available for mycotoxin analysis[16, 17]. The direct competitive ELISA

employed here was based on competition between free mycotoxin in the sample extract and mycotoxin-specific solid phase antibody. Summarized in Table 5, three out of the 65 rice samples were positive for aflatoxin B1 by ELISA and showed small amount of aflatoxin B1. Four out of 116 samples were positive for aflatoxin B1 and showed 3.4% aflatoxin B1 contamination. In a resultant value, we could mentioned that the contamination of aflatoxin B1 by *Aspergillus flavus* in Youngnam districts was still within the limits of FDA action level but more study on mycotoxin was needed.

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국문요약

영남지방 곡류에 오염되어 있는 aflatoxin B1을 측정하기 위해 신속하고 정확한 Enzyme-Linked Immunosorbent Assay(ELISA)법을 응용하였다. 생산된 antibody는 aflatoxin B_2와 강한 cross reaction을 보였고, 정량적으로 표준곡선을 보인 aflatoxin B1 범위는 1-100 ppb이었으며, methanol-PBS-dimethylformamide로 spike시킨 쌀 추출물이 ELISA 반응에 약간의 간섭반응을 보였다. ELISA법에 의한 쌀 65종, 보리 116종의 aflatoxin 분석결과는 다음과 같다. 즉, 쌀시료 65개 중 3개의 시료가 positive 반응을 보였으며, 그 중 R-18이 7.5 μg/kg, R-30이 3.6 μg/kg, R-59가 3.3 μg/kg 순으로 aflatoxin B1 함량을 나타내었고 시료의 4.6%가 aflatoxin B1에 오염되었음을 보였다. 보리 116종 중 겉보리에서 4개의 시료가 positive 반응을 나타내었으며, 그 중 겉보리 UB-37이 9.6 μg/kg의 함량으로 가장 높은 함량을 나타내었고, 그 외 UB-35, UB-15, UB-54 순이었으며 전체 시료의 3.4%가 aflatoxin B1에 오염되었음을 보였다.

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