Detection of Aflatoxin B₁ in Imported Food Products into Japan by Enzyme-Linked Immunosorbent Assay and High Performance Liquid Chromatography

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Abstract. In order to detect the presence of aflatoxin B₁ (AFB₁), the use of the enzyme-linked immunosorbent assay (ELISA) and recovery test was evaluated. The detection limit of ELISA for AFB₁ was 1 µg/assay and the recovery from maize spiked with AFB₁ exceeded 80%. AFB₁ was detected by ELISA in seven out of twelve samples of imported food products including peanut, almond, red pepper, cocoa bean, black pepper, buckwheat, walnut, adlay, soybean, popcorn, and pistachio nut, and by high performance liquid chromatography (HPLC) in four of the samples. However, the content of AFB₁ in these samples was less than 10 ng/g of the minimum value authorized by the Japanese sanitation law. These results demonstrate that ELISA is more sensitive than HPLC and imported food products are broadly contaminated with AFB₁—key words: aflatoxin B₁, enzyme-linked immunosorbent assay.

Aflatoxins are highly toxic and carcinogenic mycotoxins, and natural contamination of maize with aflatoxins has been reported [2, 6]. In Thailand, the contamination of agricultural products with aflatoxins is a serious economic problem [2, 6]. In the U. S. A., preharvest aflatoxin contamination has been well documented [12]. Maeda [10] examined imported raw shelled peanuts into Japan for the detection of aflatoxin B₁ (AFB₁) and reported that 33% of the Spanish type samples of peanuts imported from Brazil was contaminated with AFB₁. Until now, high performance liquid chromatography (HPLC) has been used for the detection of AFB₁ and its detection limit exceeded 100 pg [6]. In general, a good method for the detection of aflatoxins should be very specific, sensitive, and relatively simple to operate. Preferably, it should be adaptable to automation.

Recently, ELISA [13, 14, 15] has been proved an effective method for the detection of AFB₁ in food products because of its sensitive and simplicity. Therefore, attempts were made to evaluate the use of ELISA method for the detection of contamination of food products with AFB₁. A comparison of the results obtained by ELISA and HPLC showed that ELISA for the detection of AFB₁ can be more sensitive than HPLC and that imported food products are broadly contaminated with AFB₁.

Materials and Methods

Aflatoxins used: Aflatoxin B₁, B₂, G₁ and G₂ used in this test were purchased from Sigma Chemical Company (St. Louis, U. S. A.).

Imported food products examined: Twelve samples including peanut, almond, red pepper, cocoa bean, black pepper, buckwheat, walnut, adlay, soybean and pistachio nut supplied by the Mycotoxin Research Association (92, Yamashita-cho Naka-ku, Yokohama-Shi, Japan) were examined in this experiment. A maize sample which is not contaminated with AFB₁ was kindly supplied by National Institute of Animal Industry.

Preparation of anti-AFB₁ antibody and AFB₁-peroxidase conjugate as antigen for ELISA: The preparation of AFB₁ O-carboxymethylxime was carried out as previously described [8]. A solution of AFB₁ and O-carboxymethylhydroxylamine hemi hydrochloride in 4 M ethanol and 2 M aqueous NaOH was refluxed for 3 hr. After standing at 25°C overnight, the mixture was concentrated. Water was added, the pH was adjusted to 9.5 with 1N NaOH, and the solution was extracted with ethyl acetate. The aqueous layer was acidified to pH 2 with 6 N HCl and stored at 0°C overnight for the production of oxime as yellow precipitate. The precipitate was collected by centrifugation and dried in vacuum over anhydrous CaSO₄. The product was stored in 1.0 m/
dimethyl sulfoxide (DMSO, Wako Pure Chemical Industry Ltd., Osaka, Japan) at −10°C.

The O-carboxymethylxime of AFB_{1} was coupled to bovine serum albumin (BSA, Sigma Chemical Company, St. Louis, U. S. A.) for the preparation of the antigen inoculated into rabbits [5, 13]. After the reaction, the mixture was dialyzed against 3 liters of distilled water and changed daily for 5 days [5].

In order to prepare the antigen for ELISA, peroxidase (Type VI, Sigma Chemical Company, St. Louis, U. S. A.) instead of BSA was used for conjugation and the reaction for conjugation with AFB_{1} was carried out as previously described [13].

**Extraction and cleanup of AFB_{1} from food products:** Extraction and cleanup of AFB_{1} from samples homogenized (homogenizer: Nihon Seiki Kaisha Ltd., Tokyo, Japan) were carried out according to the method of Minamizawa et al. [9]. Samples were milled at the level of 10 mesh and made uniform. One gram of the milled food product was used for the survey of AFB_{1}.

**ELISA:** ELISA was carried out as previously described [14]. The fluid in a well was transferred into polystyrene plate for reading the absorbance at 490 nm (EIA reader, BIO RAD Chemical Division, California, U. S. A.). The colorimetric value obtained by ELISA was converted to a quantitative value of AFB_{1} which was calculated on the basis of the standard curve of AFB_{1} obtained by ELISA. This test was repeated 3 times and the value was expressed as geometric mean.

**HPLC:** Sample extracted and cleaned was dried with a rotary evaporator (Yamato Kagaku Co., Ltd., Tokyo, Japan) and redissolved in benzene-acetonitrile (98:2). Ten microliter of the sample was injected into an HPLC (Shimadzu Corporation, Kyoto, Japan). A stainless steel column (Shim-pack CLC-SIL4, Shimadzu Corporation, Kyoto, Japan) was used and fluorescence HPLC monitor RF-535 (Shimadzu Corporation, Kyoto, Japan) was used for the detection of aflatoxins (365 nm excitation and 425 nm emission). Toluene-ethylacetate-formic acid-methanol (89:7.5:2.0:1.5) was used as the mobile phase for the analysis of AFB_{1} [7].

**RESULTS**

**Optimum conditions of ELISA for the detection of AFB_{1} by ELISA:** In order to detect AFB_{1} by ELISA, an antibody dilution that gives 80% of the

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Table 1. Recovery test of AFB_{1} from spiked maize

<table>
<thead>
<tr>
<th>AFB_{1} added (ppb)</th>
<th>HPLC^{a)}</th>
<th>ELISA^{b)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.00</td>
<td>84.90</td>
<td>96.56</td>
</tr>
<tr>
<td>20.00</td>
<td>83.13</td>
<td>93.29</td>
</tr>
<tr>
<td>40.00</td>
<td>81.18</td>
<td>74.78</td>
</tr>
</tbody>
</table>

^{a)} HPLC: high performance liquid chromatography.
^{b)} ELISA: enzyme-linked immunosorbent assay.

This test was repeated two times and the values showed a geometric mean.

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**Fig. 1.** Standard curve of aflatoxin B_{1} by ELISA.

**Fig. 2.** The specificity of anti-AFB_{1}-BSA conjugate serum.
Table 2. The detection of AFB₁ in imported food products in Japan

<table>
<thead>
<tr>
<th>Food products examined</th>
<th>AFB₁ detected (ng/gram)</th>
<th>HPLC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ELISA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut 1</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Peanut 2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Almond</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Red pepper</td>
<td>2.0</td>
<td>4.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Cocoa bean</td>
<td>0.6</td>
<td>2.7</td>
<td>ND</td>
</tr>
<tr>
<td>Black pepper</td>
<td>10.8</td>
<td>6.5</td>
<td>ND</td>
</tr>
<tr>
<td>Buckwheat</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Walnut</td>
<td>ND</td>
<td>0.7</td>
<td>ND</td>
</tr>
<tr>
<td>Adlay</td>
<td>ND</td>
<td>0.3</td>
<td>ND</td>
</tr>
<tr>
<td>Soybean</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Popcorn</td>
<td>ND</td>
<td>0.5</td>
<td>ND</td>
</tr>
<tr>
<td>Pistachio nut</td>
<td>4.6</td>
<td>4.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

- HPLC: high performance liquid chromatography.
- ELISA: enzyme-linked immunosorbent assay.
- ND: not detectable.
- Geometric mean of the values given by ELISA.

with each 10, 20, or 40 ppb of AFB₁ were carried out and AFB₁ was detected by both ELISA and HPLC. The content amounted to over 80% of AFB₁ except for one value of ELISA (Table 1) and the value obtained by ELISA tended to be higher than that of HPLC. However, the difference between the values of ELISA and HPLC was not significant (p<0.05).

The specificity of anti-AFB₁ serum: The specificity of anti-AFB₁ serum was examined and the result was shown in Fig. 2. The serum to AFB₁-BSA conjugate reacted most specifically with AFB₁ but the specificity of its serum to AFG₂ was lowest in these aflatoxins.

Survey for the detection of aflatoxin B₁ in food products using ELISA and HPLC: As shown in Table 2, 12 samples including 11 different kinds of food products imported in 1985 were analysed for the detection of AFB₁ by ELISA and HPLC. By ELISA, AFB₁ was detected in seven out of the samples. Especially, the content of AFB₁ in red pepper, cocoa bean, black pepper and pistachio nut exceeded 1 ng/g of AFB₁. By HPLC, AFB₁ was also detected in these four samples. However, other three samples of which AFB₁ content was determined less than 1 ng/g by ELISA did not show AFB₁ content by HPLC. The detection limit of HPLC was 0.5 ng/g.

DISCUSSION

Aibara [1] emphasized the need for developing an immunological technique as a rapid method for the detection of AFB₁ in food products. In order to ensure the safety of food products and the health of people, we attempted to detect AFB₁ in food and feed products by using ELISA which was more sensitive than HPLC. ELISA has, so far, been carried out under the condition that the AFB₁-BSA conjugate as an antigen is fixed on the bottom of the well of ELISA plate. In this case, the quantity of the antigen fixed on the bottom of the well was not constant. Therefore, the method could be unsuitable for the quantitative analysis of AFB₁ [3, 4, 11]. On the other hand, Ueno et al. [14, 15] proposed improved ELISA method for the quantitative analysis of AFB₁. They used a filtration plate instead of a polystyrene plate. The constant amount of antigen, antiserum and immunobead was determined by the preliminary experiment in order to perform the competitive ELISA. This ELISA can be suitable for the quantitative analysis since the constant amount of antigen, antiserum and immunobead are used.

In this experiment, we first evaluated the sensitivity of the reaction between antigen and antibody in order to identify the optimum condition for the detection of AFB₁ by ELISA. The ELISA test was thus carried out based on the conditions previously described [14, 15]. The detection limit was improved in 1 pg/assay. The sensitivity was higher than that reported previously [14]. The specificity of the serum to AFB₁-BSA conjugate was also examined and its specificity to AFB₁ was considerably high as compared with the others. On the other hand, the results of the recovery test demonstrated that the value of ELISA tended to be higher than that of HPLC. Our results showed that seven out of twelve samples were contaminated with AFB₁ and the content in each sample was less than 10 ppb.

Usually, food products contaminated with more than 10 ppb of AFB₁ cannot be imported according to the Japanese food sanitation law and such food products are designated as "positive". Maeda [9] also detected aflatoxins in raw shelled peanut samples belonging to the Virginia and Spanish types and reported that 0.5% out of 2612 Virginia type samples and 5% of 9412 Spanish type samples were "positive". Until now, HPLC has been used for the detection of AFB₁. The sensitivity is comparatively low, the equipment is very expensive and the running cost is very high. A good method should be very specific, sensitive, and relatively simple to operate. Recently, ELISA which satisfies such
conditions has been developed for the detection of AFB$_1$ [14, 15] with a detection limit of 10 pg/assay.

Furthermore, to achieve a greater sensitivity, a cleanup step was added before ELISA was performed. Minamizawa et al. [9] reported the procedure for extraction and cleanup of AFB$_1$ from food products which led to a recovery of over 80%. Therefore, these techniques were used in the subsequent experiments for the detection of AFB$_1$. In conclusion, it was found that the imported food products examined in this test were moderately contaminated with AFB$_1$ and seven samples could be detected by ELISA while only four out of seven were also detected by HPLC. These results demonstrate that both methods are useful for the detection of AFB$_1$ and that ELISA is more sensitive than HPLC.

REFERENCES