Limited survey for aflatoxin contamination of polished rice imported into Japan

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Summary

Analysis of aflatoxins (AF) B₁, B₂, G₁ and G₂ in imported polished rice samples from Thailand, Pakistan and Bangladesh was performed by direct competitive ELISA (direct cELISA) and immunoaffinity column with high performance liquid chromatography (IAC-HPLC) methods using monoclonal antibody. Direct cELISA method with the detection limit of 2.8 µg/kg for AFB₁ gave negative results in all of 20 samples analyzed. On the other hand, using IAC-HPLC with the detection limit of 0.1 µg/kg for each toxin, AFB₁ was detected in five out of 20 (25%) imported polished rice samples; in three Thailand and two Pakistan samples, with the concentrations ranging 0.1 - 0.3 µg/kg. In addition, AFB₂ (0.1 µg/kg) was detected in one of the Pakistan rice sample contaminated with AFB₁ (0.3 µg/kg). Even though the level of AF contamination in imported rice samples found to be well below the Japanese limit (10 µg/kg), AF contamination in rice as a staple food of Asian is an important issue for evaluation of human exposure due to the frequency of intake.

Key words: aflatoxins, ELISA, immunoaffinity column, HPLC, imported polished rice

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Introduction

Naturally occurring aflatoxins (AF) have been classified as Class I human carcinogens and well recognized as a serious food contaminants in the tropic more than in temperate zones of the world. There is a great concern over the regulatory control of AF worldwide, and many developed countries have set stringent regulatory demands on the level of AF permitted in foods and agricultural commodities. European Union (EU) has set the regulatory limit of 2 µg/kg for AFB₁ and 4 µg/kg for total AF (AFB₁ + AFG₁ + AFB₂ + AFG₂), respectively, whereas the limit of Japan is 10 µg/kg for AFB₁.

In terms of dietary intake, contamination level of AF in corn as a staple food of most temperate countries and other corn-producing countries has been well documented. On the other hand, the contamination of AF in rice as a staple food of Asian has not been well studied and relevant data is very limited. Even though the available data indicated that the incidence and level of AF in rice are relatively low, the precision and detailed information on such contamination in staple food is considered to be prerequisite to evaluate accurately the human exposure to AF due to the frequency of intake.

The availability of a sensitive and reliable method is a key factor to detect a low level of AF in
rice. Previous data on the contamination of AF in foods for most of Asian countries, including in rice, was using thin layer chromatography (TLC) method with a relatively high detection limit. Hence, the frequency of incidence of AF in rice might have been overlooked. In Japan, Tabata et al.\textsuperscript{4} reported a mean level of 2.7 µg/kg for AF in rice using TLC method with the detection limit of 1 µg/kg.

Recently, immunoaffinity column (IAC) clean up of sample extract coupled with high-performance liquid chromatography (HPLC) with fluorescence detection\textsuperscript{5} has become popular as a sensitive and reliable method to determine a low level of AF contamination in foods. In the present study, in-house IAC-HPLC method using monoclonal antibody (mAb) with a detection limit of 0.1 µg/kg for each AF was applied to determine AF in polished rice samples imported into Japan. In-house direct competitive ELISA (direct cELISA) using mAb with a detection limit of 2.8 µg/kg for AFB\textsubscript{1} was also applied as a possible alternative method.

Materials and Methods

Reagents

AF standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and their standard stock solutions were prepared according to the AOAC method\textsuperscript{6}. All the reagents except specified were purchased from Wako Pure Chemical Industries Ltd. (Osaka). All organic solvents used for sample extraction were of reagent grade and distilled in glass apparatus before use, and the solvents for HPLC were of HPLC grade. Ovalbumin (OVA; grade III, A-5378) was purchased from Sigma Chemical Co. PBS/Tween-20 solution consist of 10 mM phosphate buffer saline pH 7.4 (PBS) containing 0.14 M sodium chloride and 0.05 % (v/v) Tween-20.

Hybridoma cell lines

Hybridoma cell lines were provided by National Center for Genetic Engineering and Biotechnology (NCGEB), National Science and Technology Development Agency (NSTDA), Ministry of Science, Technology and Environment, Thailand. Two clones of stable hybridoma cell lines secreting immunoglobulin G (IgG) anti-AFB\textsubscript{1} mAbs namely AF5 and AF8 were prepared from spleen of BALB/c mice. All of mAbs prepared were studied for cross reactivity by in-house direct cELISA. Taking the cross reactivity against AFB\textsubscript{1} as 100 % the mAb AF5 was reactive against AFG\textsubscript{1}, AFB\textsubscript{2} and AFG\textsubscript{2} as much as 68, 11 and 2 % respectively. On the other hand, the mAb AF8 was reactive against AFG\textsubscript{1}, AFB\textsubscript{2}, and AFG\textsubscript{2} as much as 88, 63 and 6 % respectively. Therefore, mAb AF5 was chosen in direct cELISA for determining AFB\textsubscript{1} because of its lower cross reactivity against the other AF, while the mAb AF8 was chosen in IAC-HPLC for its wide range of specificity.

Samples and extraction

In December 2002, 20 imported polished rice samples (0.5 - 1 kg each) from Bangladesh (1), Pakistan (2) and Thailand (17) were purchased from supermarkets in Kobe, Takamatsu, and Tokyo, Japan. Individual rice samples (200 g each) were ground with the Wonder blender (Iwaki Co. Ltd., Tokyo) to fine powder, sieved through a 0.5 mm size siever, and subsequently stored in zip-lock plastic bags at -20 °C prior to analysis. The ground samples of 5 g were extracted with 25 ml of methanol-water (55:45, v/v) by shaking for 30 min, centrifuged (1,400 x g, 10 min), and diluted to a final concentration of 10 % methanol with PBS/Tween-20 and PBS for direct cELISA and IAC-HPLC methods, respectively (Fig. 1). First, the diluted extracts were analyzed by direct cELISA method for AFB\textsubscript{1} and then by IAC-HPLC method for total AF. All experimental treatments for each subsample were performed in triplicate.
**Direct cELISA method**

The mAb AF5 (5 µg/ml) was added to each well of 96-well ELISA plate Catalog No. 442404 (Nalge Nunc International, Roskide, Denmark) and incubated at 4 ºC overnight. The coating solution was removed from the plate by washing 3 times with 150 µl PBS/Tween-20. The wells were then filled with 150 µl of 0.1 %OVA-PBS, incubated at room temperature for 1.5 hr to block the unoccupied sites, and the plate was washed 3 times as mentioned above. The 50 µl of standard AFB1 solution (or diluted extracts in 10 %methanol - PBS/Tween-20) and 50 µl of AFB1-horseradish peroxidase conjugate in PBS/Tween-20 (0.66 µg/ml) were then added, and incubated for 1 hr at room temperature followed by washing 5 times as mentioned above. The 100 µl of 2,6,10,14-tetramethylbenzidine solution (0.1 mg in 15 ml of 0.1 M acetate buffer, pH 5.0, containing 0.005 % H2O2) was added to each well, incubated at room temperature for 40 min, and the reaction was terminated by addition of 50 µl of 1 M H2SO4. The absorbance reading at 450 nm using 620 nm as a reference absorbance was determined in an ELISA reader (Nalge Nunc International).

**IAC-HPLC method**

IAC preparation of Ueno et al.7 was modified. Briefly, 7 ml of Affi-Gel-10 (Bio-
Rad Laboratories, Hercules, CA, USA) was washed with 14 ml distilled water and filtered through 3G-3 glass filter followed by placing 3 ml of Affi-Gel-10 to a new tube. The mAb AF8 purified by 50 % saturated ammonium sulfate precipitation (8.0 ml, 80.56 mg) was immediately added and gently mixed by hand for 1.5 hr. After filtering through 3G-3 glass filter, the gel was then blocked with 4 ml of 1 M ethanolamine pH 8.0 at room temperature for 1 hr, and washed with 21 ml PBS. The 0.2 ml immobilized gel was suspended with PBS containing 0.1 %sodium azide in a mini-column (1cm i.d. × 3 cm) and kept at 4 °C until used. As shown in Fig. 1, the 20 ml of diluted sample was applied onto the IA column at a flow rate of 1 ml/min (by gravity), washed with 10 ml of distilled water and enough air was applied to completely remove the water. The analyte was then eluted with 5 ml (preferably by adding 20 µl 0.25 ml) of acetonitrile followed by concentration to dryness using centrifugal concentrator (Taitec VC-36N centrifugal concentrator, and Taitec VA-500 F Freezer trap, Saitama; speed x6, temperature at 40 °C). AF was pre-derivatized with trifluoroacetic acid (TFA) and analyzed by HPLC according to the procedure of Akiyama et al.9), for which the week fluorescent AFB1 and AFG1 were converted into their strong fluorescent hemiacetals AFB2a and AFG2a, whereas the strong fluorescent AFB2 and AFG2 were not affected by this derivatization. The 100 µl of TFA was added to a dried sample, capped and mixed with a vortex mixer for 15 sec. The solution was kept at room temperature under dark condition for 15 min, then 900 µl acetonitrile-water (1:9, v/v) was added and again mix for 15 sec with vortex mixer. The solution (20 µl) was injected onto the HPLC column. A PU-980 pump, AS-950 autosampler (JASCO, Tokyo) equipped with 100 µl loop and RF-10A XL spectrofluorometer (Shimadzu Co. Ltd., Kyoto) were used for the quantitation of AF under the following conditions: column Capcell Pak C18 UG120, particle size 5 µm, 250 × 4.6 mm (Shiseido Fine Chemicals, Tokyo), oven temperature 40 °C, wavelength 450 nm for emission and 365 nm for excitation, and mobile phase methanol-acetonitrile-water (10:25:65, v/v/v) at a flow rate of 1 ml/min.

**Recovery**

Recoveries study was done by spiking polished AF-free rice samples with AFB1 at the level of 5, 10, 20, and 50 µg/kg for direct cELISA and with each of AFB1, AFG1, AFB2, and AFG2 at 0.2 and 0.5 µg/kg for IAC-HPLC methods, respectively. Spiked samples were left at room temperature under dark condition overnight and then analyzed as described above. The calibration graph was done by using standard solutions, dried and derivatized as above in the range of 0 - 55 µg/kg for direct cELISA and 0-28 µg/kg for IAC-HPLC methods.

**Results and Discussion**

The recovery of AFB1 spiked at 5, 10, 20, and 50 µg/kg were 84, 87, 90, and 95 % respectively, with the detection limit of 2.8 µg/kg in direct cELISA method. The detection limit was linear in the range of 0 - 2 ng/ml, and intra- and interassay precision were between 0.1 to 5 % and 2 to 9 % respectively. The detection limit of 2.8 µg/kg for AFB1 was better than those of AOAC9) (20 µg/kg). By this method, all of the samples analyzed were negative for AFB1 (below detection limit). However, this method has been applied successfully for determining AFB1 in corn and peanut samples in our previous study and has been proved to be convenient and reliable for screening a large scale of samples at a higher level of AFB1 contamination. In addition, it is possible to further reduce its detection limit in future studies.
As for IAC-HPLC method, the recovery of AFB₁, AFG₁, AFB₂, and AFG₂ spiked at 0.2 and 0.5 µg/kg were 95, 101, 112, 110 and 82, 79, 89, 83 % respectively. The extraction solvent of 55 % methanol was found to be as efficient as 60 - 80 % methanol described by other references. The detection limit of 0.1 µg/kg for each toxin was similar to the IAC-HPLC method reported by Stroka et al., and better than those of Truckssess et al., and Urano et al. which were 5, and 0.5 µg/kg, respectively.

Using the IAC-HPLC method, AFB₁ was detected in five out of 20 (25 %) imported polished rice samples with concentrations ranging 0.1 - 0.3 µg/kg. The samples contaminated with AF were imported from Pakistan (2/2) and Thailand (3/17). In addition, AFB₂ (0.1 µg/kg) was detected in one of the Pakistan rice samples contaminated with 0.3 µg/kg AFB₁ (Table 1).

The results of AFB₁ in positive samples were qualitatively determined by comparing the HPLC chromatograms of the TFA-derivatized and underivatized sample extracts. The absence of AFB₂ₐ peak in underivatized sample extracts eliminates the possibility of other fluorescing materials giving a peak at the same retention time as AFB₁ (AFB₂ₐ) and verifies the positive results, as shown in Fig. 2. The reliability of IAC-HPLC method for determining a low level of AF in rice samples was also studied as shown in Table 1. The consistent results were obtained for AFB₁ and AFB₂ concentrations in terms of reproducibility and repeatability, indicating the reliability of this method for determining a very low level of AF contamination in rice.

In Japan, the subject of mycology and AF contamination in rice has been pointed out as early as 1957, however, accurate data using a reliable and sensitive method such as IAC-HPLC method so far is not available. Result of the present study is in agreement with those reported by Tabata, for which AF was not found in domestic Japanese rice samples (n=5) analyzed using the same IAC-HPLC method described in this study. Thus, it is also suggested that the AF contamination of polished rice in Japan was resulted from imported rice, particularly from the countries of tropic climate.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Country</th>
<th>Analyze No.</th>
<th>AFB₁ Concentration (µg/kg)</th>
<th>AFB₁ Mean</th>
<th>AFB₂ Concentration (µg/kg)</th>
<th>AFB₂ Mean</th>
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*Not detected (below the detection limit, 0.1 µg/kg)

All experimental treatments for each subsample were performed in triplicate.
In conclusion, the IAC-HPLC method has been successfully applied as a sensitive and reliable method to determine the level of AF in rice near to the detection limit (0.1 µg/kg). Even though the level of AF contamination in imported polished rice in Japan was found to be low, well below the limit of 10 µg/kg, the frequency of intake of staple food should be taken into consideration for the accurate risk assessment. This is the first report on a limited survey of AF contamination in imported polished rice in Japan using both direct cELISA and IAC-HPLC methods.

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輸入白米のアフラトキシン汚染事例

スウィウェク・リビゴルゴソニ、ノルハヤティ・アリ、芳澤宅實：香川大学農学部（761-0795 香川県木田郡三木町池戸）

タイ、バキスタン、バングラデシュからわが国に輸入された白米を汚染するアフラトキシン（AF）の分析を、モノクローナル抗体を用いた直接競合ELISA法ならびにイムノアフィニティーカラムとHPLC（IAC-HPLC）法により行なった。ELISA（検出限界2.8 µg/kg）では、分析した20検体からアフラトキシンB₁（AFB₁）は検出されなかった。しかし、IAC-HPLC（検出限界0.1 µg/kg）においては、20検体中5検体（タイ3検体、バキスタン2検体）から0.1-0.3 µg/kgのAFB₁が検出され、またAFB₁:0.3 µg/kgで汚染したバキスタン米からはAFB₁(0.1 µg/kg)も認められた。輸入米のAF汚染レベルはわが国の基準値（10 µg/kg）に比べてきわめて低いレベルであったが、アジア地域の主食としてのコメのAF汚染はヒトのAF暴露を精確に評価する上で重要な課題である。

キーワード：アフラトキシン、ELISA、イムノアフィニティーカラム、HPLC、輸入白米