Occurrence of aflatoxins in processed chili pepper sold in Myanmar

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Keywords

aflatoxin B₁; aflatoxin B₂; chili; immunoaffinity column

Abbreviations

chili pepper, CP; aflatoxin, AF; immunoaffinity column, IAC; high-performance liquid chromatography, HPLC

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(Received December 12, 2018, revised January 9, 2019, accepted January 18, 2019)

Abstract

A limited surveillance of aflatoxins (AFs) contamination was conducted with ten samples of processed chili pepper (CP) retailed in commercial market in Myanmar during 2017. All samples were dried and ground for consumption. The concentrations of major AFs, aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), and aflatoxin G₂ (AFG₂), in these samples were quantitatively analyzed by high-performance liquid chromatography-fluorescence (HPLC-FL) detection after immunoaffinity column purification. Each value was confirmed by spike and recovery test. The contamination of AFB₁ was detected in nine samples except for one sample (CP-3). That of AFB₂ was detected in four samples (CP-6, CP-7, CP-8, and CP-9). One sample with the highest concentration of AFB₁ and AFB₂ (CP-8) showed the value of total AFs (sum of major four AFs) above 10 µg/kg, while others showed below 10 µg/kg, the maximum permissible limit of total AFs level set by many countries. No contamination of AFG₁ and AFG₂ was found in ten samples. The data obtained here suggest that CP may have affinity to *Aspergillus flavus*, a B-type AFs producer.

Introduction

Mycotoxins are toxic chemical substances produced by a variety of fungi which contaminate food^{1),2)}. Toxicity due to mycotoxins is almost always insidious, without any overt indication of effects on health in the short terms. For this reason, the health effects of mycotoxins are among the most neglected areas of medical science³⁾. Mycotoxin contamination is not only a cause of health hazards to humans and animals but is also a global problem for the agricultural economy. The Food and Agriculture Organization (FAO) of the United Nations estimates that 25% of the world's food crops are affected by mycotoxins, of which the most notorious are aflatoxins (AFs)⁴⁾.

Among mycotoxins, AFs are hepatocarcinogenic and being the most toxic mycotoxins mainly produced by some aflatoxigenic strains of *Aspergillus flavus* and

A. parasiticus. The most common AFs are aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), and aflatoxin G₂ (AFG₂), which often co-occur in food and represent total AFs. They are among the most potent mutagenic and carcinogenic compounds known to be produced in nature. AFs contamination caused by aflatoxigenic fungi is the most serious issue for safety throughout the world, and various agricultural commodities have been found to be contaminated with aflatoxigenic fungi and/or AFs. Although the presence of *Aspergillus* fungi does not necessarily indicate AFs contamination, there is certainly an increased risk⁵⁾. Aflatoxigenic fungi are known to exist in tropical/sub-tropical area, where is favorable climate condition for fungal propagation and AFs production⁶⁾. The climate and geographical location of Myanmar provide favorable conditions of the AFs contamination in agricultural commodities in the field as well as post-harvest (during

storage)⁷.

Chili pepper (CP), made from red chili (*Capsicum* spp.), is one of the most popular spices consumed in cuisines worldwide. Contamination of spices with AFs can take place in the field, during drying and during the storage and processing stages⁸. CP is mainly produced in warm regions including Southeast Asia. There are reports of CP contaminated with AFs in various countries, sometimes with high prevalence^{9,10}. However, there exists little report of AFs surveillance in spices including CP sold in Myanmar, while Myanmar people widely used CP powder for culinary purposes¹¹. In this study, we firstly analyzed the amounts of AFB₁, AFB₂, AFG₁, and AFG₂ concentrations in CP sold in Myanmar markets, using in-house validated method. Each value was measured by HPLC-FL apparatus after immunoaffinity column purification with confirmation by spike and recovery test.

Materials and Methods

Samples, materials and reagents

Ten bags of processed chili pepper (CP) were purchased at several markets in Myanmar, from April 2017 to September 2017. CP-1 was a KSS chili pepper, CP-2 was a Daw Htwe fresh and pure chili pepper, CP-3 was a Myo Ma Sin Phyu Taw coarse ground chili, CP-4 was a

Myo Ma Sin Phyu Taw chili pepper, CP-5 was a Daw Htwe fine ground chili pepper, CP-6 was a Nan Dae Wei chili pepper, CP-7 was a KSS fine ground chili pepper, CP-8 was a U Ngwe Soe chili pepper, CP-9 was a Number 1 chili pepper and CP-10 was a U Hla Tun chili pepper. They were dried and ground ones for cuisine ingredients.

An immunoaffinity column (IAC) AFLAKING was purchased from HORIBA (Kyoto, Japan). Standard aflatoxin B₁ (AFB₁), and aflatoxin G₁ (AFG₁) were purchased from Acros Organics, Geel, Belgium, while aflatoxin B₂ (AFB₂), and aflatoxin G₂ (AFG₂) were purchased from Fujifilm Wako, Osaka, Japan. They were dissolved in acetonitrile as a working solution containing 50 ng/ml of each. Acetonitrile, methanol and water were HPLC grade.

Aflatoxin analysis

For sample preparation (shown in Scheme 1), ca. 30 g of CP samples were ground and mixed in cups of blender (Waring, laboratory blender) and 2.5-5 g portions of grind samples were put into 50 ml centrifuge tubes (2 each, one for Blank, and the other for Spike). Either 250 µl of 100% acetonitrile (for Blank) or 50 ng/ml AFs mixture (AFB₁, AFB₂, AFG₁ and AFG₂) standard solution (for Spike) was added and kept at room temperature for 15 min. Then 0.5-1.5 g of sodium chloride

Weigh 2.5 - 5 g of CP powder in 50 ml centrifuge tubes (x 2 for one CP sample)

↓ add 0.25 ml of 100% acetonitrile (for blank) or AF mix (50 ng/ml)(for spike)

↓ wait for 15 min

↓ add 0.5 - 1.5 g of NaCl

↓ add 20 ml of methanol - water (4:1, v/v)

↓ vortex for 1 min and sonicate for 3 min

↓ centrifuge for 10 min at 1,470 g

Supernatant

↓ transfer 10 ml to 50 ml cylinder flask and fill up to 50 ml with water

↓ filtrate the diluted solution through a funnel pre-set filter

↓ apply 10 ml of filtrate on a reservoir set on IAC (pre-conditioned with PBS) and elute with gravity

↓ rinse the IAC with 3.5 ml of water for three times

↓ elute AF with 1 ml of acetonitrile with 5 min interval for three times

Collect all solvent by passing air through the IAC

↓ evaporate the solvent, add 0.1 ml of TFA and mix well

↓ leave for 15 min

↓ add 0.9 ml of acetonitrile - water (1:9, v/v)

↓ transfer to HPLC vial

HPCL-FL analysis

Scheme 1 Analytical method for AFs in CP

was added. They were extracted with 20 ml of methanol - water (4:1, v/v), vortexed and sonicated. After centrifuged at 1,470 *g* for 10 min, 10 ml of the supernatant was taken and filled up to 50 ml with water. The diluted solution was filtrated, and 10 ml of the filtrate was passed through an IAC AFLAKING, which was pre-conditioned with phosphate buffered saline (PBS). IAC was washed 3 times with 3.5 ml of water, then 1 ml of 100% acetonitrile was applied onto IAC 3 times to elute AFs by gravity. Collected eluents were evaporated, and 0.1 ml of trifluoroacetic acid (TFA) was added for derivatization of AFB₁ and AFG₁ to enhance their fluorescence. After kept at room temperature for 15 min, 0.9 ml of acetonitrile - water (1:9, v/v) was added, and the dissolved samples were directly injected into the HPLC-FL system (Shimadzu, Kyoto, Japan).

HPLC-FL analysis was performed using a Capcell Pak C₁₈ column of 3.0 × 250 mm with a 5-μm particle size (Osaka Soda, Osaka, Japan). The mobile phase was composed of acetonitrile, methanol, and water (10:30:60, v/v/v), and used with a flow rate of 0.3 ml/min. The column heater was set at 40°C, and the injection amount was 5 μl. The AF-TFA derivative solution was detected by FL absorption using wavelength of 365 nm (excitation) and 450 nm (emission). Six standard concentrations of AFs working solution, covering a range from 1 to 20 ng/ml (1, 2, 5, 10, 15, and 20 ng/ml), were employed, and calibration curves were plotted with peak areas against the concentration of AFs (ng/ml). The linearity of the calibration plot was demonstrated by calculating the correlation coefficient. The limit of detection (LOD) was defined as the concentration that was three times higher than the standard deviation of the blank signal. The LODs (ng/g) were 0.4, 0.2, 0.7, and 0.5 for AFB₁, AFB₂, AFG₁, and AFG₂, respectively, except for one sample (CP-8). For CP-8, the LODs (ng/g) were 0.8, 0.4, 1.4, and 1.0 for AFB₁, AFB₂, AFG₁, and AFG₂, respectively. The limit of quantification (LOQ) was set as 1.5 times higher value of LOD.

Results and Discussion

AFs amounts in CP samples were determined by in-house validated analytical method using Blank and Spike analyses as shown in Table 1. Recovery ratios were 68-124%, 84-116%, 72-126%, and 68-120% for AFB₁, AFB₂, AFG₁, and AFG₂, respectively. Most of these values were inside of the range from 70 to 120%, which is in the generally accepted range for in-house validation of analytical methods using spike and recovery tests. Although some data were outside this range, we consider that it will not matter for practical analyses because it showed a slightly lower (68%) or higher (124%, 126%) values. As for CP-7 sample, the recovery ratios were 160%, 124%, 132%, and 128% for AFB₁, AFB₂, AFG₁, and AFG₂, respectively, when 0.5 g of salt was used. On the other hand, the recovery ratios became 124%, 116%, 124%, and 120% for AFB₁, AFB₂, AFG₁, and AFG₂, respectively, when 1.5 g of salt was used for CP-7. Although the values of AFB₁ and AFB₂ were same (8.2 ppb and 0.3 ppb) under both conditions, increase of salt amount might be effective to obtain proper recovery ratio by salting-out of biopolymers. As for CP-8 sample, the recovery ratios were unstable even when the salt amounts were increased, showing higher AFB₁ value in blank sample than that in spiked sample. The reduction of sample amount to half worked well for a proper recovery ratio of AFB₁ for CP-8, presumably due to dilution of biopolymers which could interfere with antigen-antibody reaction.

As shown in Table 1, AFB₁ was detected at the concentration of more than 0.8 ppb, except for CP-3. CP-8 showed highest AFB₁ value with the concentration of 39.2 ppb (Fig. 1). AFB₂ were detected at concentration of 0.4, 0.3, 2.4, and 0.4 ppb for CP-6, CP-7, CP-8, and CP-9, respectively. On the other hand, AFG₁ and AFG₂ were not detected. In the current study of limited surveillance, one sample (CP-8) out of ten exceeded the regulatory limit for total AFs (10 μg/kg) in many

Table 1 Occurrence and recoveries of major aflatoxins in ten CP samples

Sample number	AFB ₁ (ppb)	AFB ₂ (ppb)	Recovery (AFB ₁)	Recovery (AFB ₂)	Recovery (AFG ₁)	Recovery (AFG ₂)	Total AFs (ppb)
CP-1	8.6	<LOD	88%	92%	80%	68%	8.6
CP-2	2.2	<LOD	72%	100%	112%	88%	2.2
CP-3	<LOD	<LOD	92%	84%	96%	80%	<LOD
CP-4	0.8	<LOD	112%	116%	72%	80%	0.8
CP-5	1.2	<LOD	68%	104%	96%	88%	1.2
CP-6	7.5	0.4	120%	112%	124%	120%	7.9
CP-7*	8.2	0.3	124%	116%	124%	120%	8.5
CP-8**	39.2	2.4	77%	95%	126%	112%	41.6
CP-9	8.5	0.4	92%	100%	112%	116%	8.9
CP-10	1.5	<LOD	88%	108%	104%	104%	1.5

*1.5 g of salt was used.

**2.5 g of sample was used.

Fig. 1 HPLC-FL chromatogram of
 (a) Extract of CP-3 (non-spiked)
 (b) Extract of CP-3 (spiked)
 (c) Extract of CP-8, a sample with natural contamination

countries including European Commission. More extensive study will be needed in the follow-up research.

In previous studies in other countries, high prevalence of natural contamination of CP with AFB₁ was reported. For example, 77% in Sri Lankan CP¹², 100% (10 out of 10) in Iranian red pepper¹³, and 96.7% (29 out of 30) in Thailand CP¹⁴. In most cases, besides

AFB₁, AFB₂ was sometimes simultaneously detected, while AFG₁ nor AFG₂ was not detected, as in the case of this study.

It is widely recognized that CP is susceptible to *A. flavus*, an aflatoxigenic species of *Aspergillus*, which is a typical B-type AFs producer¹⁵. There is no information of fungal species responsible for AFB₁ and AFB₂ contam-

ination of Myanmar CP found in this study, however; *A. flavus* adherence to Myanmar CP is strongly suggested since B-type AFs were exclusively detected in nine of ten CP samples from various markets. Soil and air are the main inoculums for causing contamination in crude spices in field¹⁶. Even where good manufacturing practices have been followed, AFs are considered unavoidable contaminants of food and feed. Spices are collected in tropical areas using traditional methods, which mean products are exposed to contaminants from the soil and air, before being sufficiently dry to prevent microbial growth¹⁶, as well as during harvesting, handling and packing. Further studies to identify fungal species adherent to Myanmar CP should be conducted to elucidate the source of AFs contamination.

Acknowledgements

This work was supported by JICA-KIRIN fellowship program (2017-2018), and partially supported by JSPS KAKENHI Grant Number JP16H05785. We are grateful to Dr. Yoshitsugu Sugiura for his valuable supervision, Dr. Masahiro Nakajima: Manager, Food Department of Nagoya City Public Health Research Institute, and Dr. Mikiko Uchigashima: HORIBA, Ltd. for valuable advice. We are also grateful to Ms. Hidemi Hatabayashi and Ms. Annette Monigina Nyangaresi for technical support.

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