研究ノート

Preparation of an in-house reference material of Thai rice containing citrinin

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Abstract

Less is known about the contamination of rice with mycotoxins than that of wheat or corn. However, the post-harvest contamination of rice with aflatoxin, sterigmatocystin, or citrinin does occur occasionally during storage and transportation. In this study, we prepared a reference material containing citrinin by applying the method used for the preparation of reference materials containing sterigmatocystin. An artificially citrinin-contaminated Thai rice sample was prepared and mixed with blank Thai rice in a sealed container. The homogeneity of the resulting mixture was confirmed by one-way analysis of variance, which confirmed that this mixture is a suitable in-house reference material.

Key words: mycotoxin, *Penicillium*, homogeneity, sterigmatocystin

Introduction

Rice is a staple food in many countries in Asia. Although rice is generally more tolerant to fungi than wheat and corn, *Aspergillus* and/or *Penicillium* species are occasionally detected in rice during storage and transportation[1)].

The most toxic group of mycotoxins produced by *Aspergillus* species is aflatoxins, for which regulatory limits exist in many countries[2)]. Several years ago, aflatoxin B_1 contamination in imported rice became a social problem in Japan[3)]. As a result, to strengthen aflatoxin monitoring, after 2011, the regulation on aflatoxin B_1 (10 ppb) was modified to a regulation on total aflatoxins (i.e., the sum of aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, and aflatoxin G_2) in Japan. A proficiency test material for aflatoxins—fapas—is now available commercially (http://fapas.com/; total aflatoxins in rice). Another *Aspergillus* toxin occasionally found in rice is sterigmatocystin, a biosynthetic intermediate of aflatoxins. Indeed, rather high contamination of rice with sterigmatocystin (16.3 ppm) has been reported in a Japanese rice storehouse[4)]. Currently, no proficiency test materials are available for the validation of analytical methods for the determination of sterigmatocystin in Japanese rice, although Tanaka et al. (2008) prepared an in-house reference material previously[5)].

In contrast, *Penicillium* species adhering on rice are known to produce other mycotoxins, which are historically termed yellow(ed) rice toxins: citreoviridin, luteoskyrin, cyclochlorotine, and citrinin[6)]. Citrinin is a nephrotoxic mycotoxin, and its toxicity is enhanced when it co-occurs

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with ochratoxin A[7]. Citrinin exists in rice food fermented by *Monascus purpureus* (*beni-kouji*), and the EU has set a maximum residue level of citrinin in fermented rice supplements at 2 ppm ((EU) No 212/2014, 2014)[8]. *Penicillium citrinum*, which was the species first identified as producing citrinin, often exists in paddy field soil and rice plants. Furthermore, this species appears to be rice specific and is rarely found on plants other than rice[9]. The risk of citrinin contamination of rice is serious, and a validated analytical method for citrinin in rice is required. However, currently, no reference materials of rice containing citrinin are available. In this study, we aimed to prepare an in-house reference material containing citrinin in a Thai rice matrix.

Materials and Methods

Fungal strains and culture condition

Wild-type *P. citrinum* MAFF 111019 (NARO Genebank), whose production of citrinin was confirmed on potato dextrose agar (PDA) plates, was used to inoculate blank Thai rice (commercially available white rice). This strain was cultured in a 1/2× PDA slant tube for 7 days, and a spore suspension was prepared. Ten grams of Thai rice was weighed in a 100-mL Erlenmeyer flask, wetted with 5 mL of water for 3 h, and autoclaved at 121 ºC for 20 min to serve as the citrinin-producing culture medium. This Thai rice medium was inoculated with 10 µL of spore suspension (32×10^6/mL) and kept for two weeks at 25 ºC.

Grinding and homogeneity test

Grinding was performed according to the method of Tanaka et al. (2008)[5] with slight modifications. After two weeks of culture, the Thai rice medium artificially contaminated with citrinin was autoclaved (121 ºC for 20 min) and ground with blank Thai rice grain at a 1/40 ratio (10 g of citrinin-contaminated Thai rice/400 g of blank Thai rice). Grinding was conducted in a ShakeMaster apparatus (Biomedical Sciences, Tokyo, Japan) with a 1-L container and 50 stainless-steel balls (diameter: 2 cm) to obtain a fine powder (First mixed sample, Scheme 1). In this work, the amount of sample subject to grinding was 400 g instead of 300 g, and a grinding time shorter than 60 min was used because grinding for longer periods increased the sample temperature. The particle size after grinding was measured using a particle size distribution analyzer (SALD-2100, Shimadzu, Kyoto, Japan). Dilution with blank rice was conducted to obtain 400 g of powdered sample containing a low level (<1 ppm) of citrinin (Second mixed sample, Scheme 1). The resultant powdered sample was inverted manually in a zipper bag 60 times to mix it completely before it was divided into 20 subsamples of ca. 20 g each. Two 5.0-g aliquots were taken from each subsample, and their citrinin contents were analyzed. The homogeneity of the sample was tested by one-way analysis of variance (ANOVA) via manual calculation using Microsoft Excel.

Citrinin analysis

The extraction and purification of citrinin were conducted according to the method of Aoyama with slight modifications[10]. First, 5.0 g of sample was extracted in a 50-mL centrifuge tube with 20 mL of acetonitrile-hydrochloric acid-water solution (8+1+1, v/v/v) by shaking on a shaker (Laboratory shaker, TAITEC, Japan) for 30 min. The tube was then centrifuged at 1,470 × g for 10 min, and 4 mL of

Scheme 1. Preparation of mixed samples containing citrinin.

Shake Master container (1 L)
- Layer the rice and stainless-steel balls in the following order
- ↓17 stainless-steel balls
- ↓200 g of Thai rice
- ↓17 stainless-steel balls
- ↓200 g of Thai rice
- ↓10 g of Artificially citrinin-contaminated Thai rice
- ↓16 stainless-steel balls
- Grind 15 min x 3 times = Pre-mix

Shake Master container (1 L)
- Layer the rice and stainless-steel balls in the following order
- ↓175 g of Thai rice
- ↓17 stainless-steel balls
- ↓50 g of Pre-mix
- ↓17 stainless-steel balls
- ↓200 g of Thai rice
- ↓16 stainless-steel balls
- Grind 15 min x 3 times = First mixed sample

Shake Master container (1 L)
- Layer the rice and stainless-steel balls in the following order
- ↓180 g of Thai rice
- ↓17 stainless-steel balls
- ↓40 g of First mixed sample
- ↓17 stainless-steel balls
- ↓180 g of Thai rice
- ↓16 stainless-steel balls
- Grind 15 min x 3 times = Second mixed sample

supernatant was evaporated under a gentle flow of nitrogen at 25 ºC; the volume of the remaining supernatant was less than 0.2 mL. Then, 0.1 g of sodium chloride and 2.0 mL of ethyl acetate were added, and the mixture was mixed vigorously for 1 min. The tube was centrifuged again, and 1 mL of supernatant was evaporated under a gentle flow of nitrogen at 25 ºC to produce a dry residue. This residue was redissolved in 0.5 mL of acetonitrile-water solution (1+1, v/v) and served as the sample solution for high-performance liquid chromatography-fluorescence (HPLC-FL) analysis. Citrinin was detected using an LC-10A (Shimadzu, Kyoto, Japan) equipped with a C18 Inertsil ODS-3V (250 × 4.6 μm i.d., 5-μm spherical particle size, GL Sciences, Tokyo, Japan) and an RF-10AXL fluorescence detector (Shimadzu) with excitation at 331 nm and emission at 500 nm. The mobile phase was isocratic and composed of acetonitrile-water-1% phosphoric acid (230+230+1, v/v/v).

Results and Discussion

Grinding conditions

The grinding conditions were assessed by comparing the particle size distributions obtained after 30 min of grinding (15 min × 2 times) and after 45 min of grinding (15 min × 3 times) using blank Thai rice. Because grinding for longer periods increased the sample temperature, we did not test grinding periods exceeding 45 min. Optically, no difference was observed between the samples obtained after 30 min and 45 min of grinding; however, the latter sample had a smaller particle size (146-169 μm) than the former (252-281 μm) (Fig. 1). Therefore, for further tests, we used the latter condition (45 min; 15 min × 3 times) to produce the homogeneous toxin-containing in-house reference material.

Preparation of the in-house reference material

Reference materials in which the analyte of interest is homogeneously distributed are very useful for the development of analytical methods because spike and recovery tests sometimes do not reflect extraction efficiency, especially when the analyte binds to biomolecules, such as proteins, carbohydrates, and fats. Despite the potential risk of citrinin contamination in rice, currently, no citrinin-containing reference materials are available. Previously, Tanaka et al. (2008) prepared an in-house reference material consisting of Japanese rice containing sterigmatocystin at the ppm level[5]. In this study, the applicability of their method was examined for the preparation of an in-house reference material consisting of Thai rice containing citrinin, a mycotoxin that is difficult to extract because of its negative charge. The repetition of grinding and mixing followed by dilution resulted in 400 g of the Second mixed sample, as shown in Scheme 1. The Second mixed sample was divided into 20 subsamples for further analysis. The citrinin contents measured in randomly collected aliquots (5 g each) of the subsamples were sufficient to pass the ANOVA performed to test the samples' homogeneity (Table 1). Therefore, the method described here—i.e., using a ShakeMaster apparatus and a zipper bag—was confirmed to be sufficient to meet the criteria of an in-house reference material containing citrinin

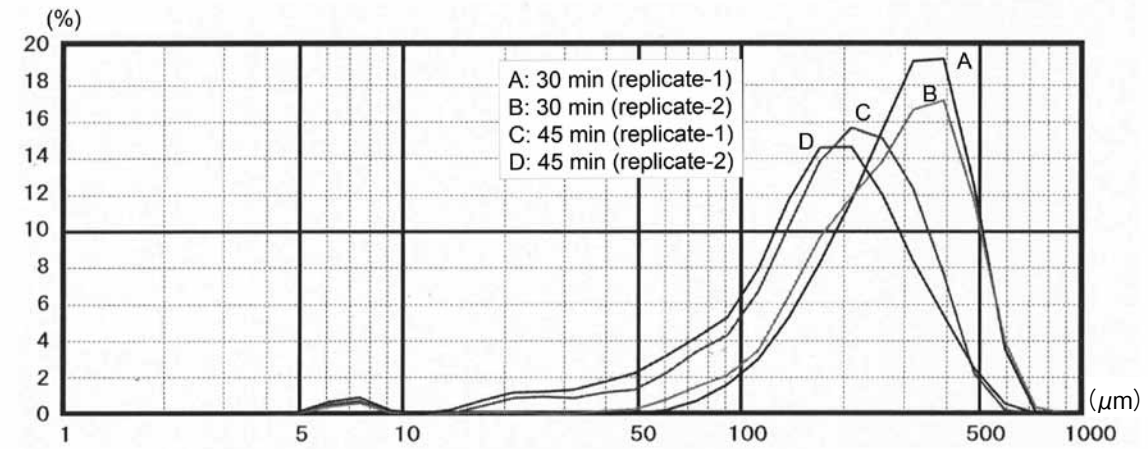

Fig. 1. Particle size distributions of the powder samples after grinding.

Table 1. Results of the homogeneity test of citrinin in the Second mixed sample

Citrinin concentration (ppm)										
ID	1	2	3	4	5	6	7	8	9	10
Replicate 1	0.1839	0.2425	0.197	0.2505	0.2023	0.2331	0.2398	0.2416	0.2621	0.1944
Replicate 2	0.2403	0.2313	0.2094	0.236	0.2445	0.2522	0.2234	0.2463	0.2514	0.212

One-way ANOVA table					
Source	Sum of squares	Degrees of freedom	Mean square	*F-value*	*p-value*
between	0.000496008	1	0.000496008	4.41387	0.33142
within	0.008960612	18	0.000497812		
total	0.00945662	19			

p-value > 0.05: Accept

at the sub-ppm level. As a result, the method of Tanaka et al. (2008)[5] can be extended to the preparation of additional in-house reference materials containing analytes other than sterigmatocystin.

Conclusion

In this study, we successfully prepared 400 g of an in-house reference material consisting of Thai rice containing citrinin at the sub-ppm level by applying the grinding and mixing procedures described by Tanaka et al. (2008)[5].

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シトリニン含有タイ米標準物質の調製

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要　旨

米のカビ毒汚染は，小麦･トウモロコシに比べ低いことが知られている。しかし，収穫後の米のアフラトキシン，ステリグマトシスティン，シトリニンによる汚染が，貯蔵・輸送中に起こることがある。本研究では，ステリグマトシスティンを含む標準物質の作製法をもとに，シトリニンを含む標準物質を作製した。人工的にシトリニン汚染したタイ米を調製し，非汚染のタイ米と，密閉容器内で混合した。混合物の均一性が一元配置分散分析により確認されたことより，インハウス標準物質として使用できる試料を得ることができた。

キーワード：マイコトキシン，ペニシリウム，均一性，ステリグマトシスティン