Mycotoxin production of some *Aspergillus ochraceus* and *Aspergillus fumigatus* isolated from the air

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Abstract

We have collected airborne fungi in houses and public facilities in Japan and currently kept about 500 strains of genus *Aspergillus*, which are the subjects of this study. From the collection, we chose *Aspergillus ochraceus* and *Aspergillus fumigatus*, which were isolated from the air during the period of 2003 - 2009, to survey their mycotoxin production by LC/MS/MS. *A. ochraceus* strains were served to investigate the production of ochratoxin A and ochratoxin B, while *A. fumigatus* strains were to examine the production of gliotoxin and verruculogen. The results confirmed that many strains are capable of producing mycotoxins. It should be noted that some airborne spores of *A. ochraceus* strains were found to produce relatively high level of ochratoxin A.

Introduction

In recent years, airborne fungi in indoor environment have been drawing attention for their influence on health. Those fungi are now known to the public particularly as a cause of allergic diseases. A report indicates a relationship between allergic diseases and fungi that the concentration of airborne fungi tends to be high (cfu/m^3) in houses with children contracting allergic diseases[1)]. A case has been also reported that the massive growth of fungi resulting from installation mistake of a exhaust fan caused excessive allergic symptoms (mold hypersensitivity)[2)].

We have surveyed indoor airborne fungi in many houses and various public facilities including art museums. They found that three fungal genera *Cladosporium*, *Aspergillus*, and *Penicillium*, dominated the microflora[3)]. In tests for identification, species known to produce mycotoxins were isolated not infrequently. Particular attention should be paid to *Aspergillus ochraceus* and *Aspergillus fumigatus*. *A. ochraceus*, which is known for the production of noted carcinogens including ochratoxin A, ochratoxin B, and ochratoxin C is

widely distributed in house environment[4,5]. *A. ochraceus* adhering onto the body surface of cigarette beetle (*Lasioderma serricorne*) has also been shown to have a high OTA producing capability[6]. *A. fumigatus*, known to produce gliotoxin, verruculogen, fumitremorgen and the like, is also the causal fungus for deep-seated mycoses, such as pulmonary aspergilloma, invasive pulmonary aspergillosis, allergic bronchopulmonary aspergillosis, and so on[7]. All such mycoses develop when airborne spores are inhaled. Because mature colonies of *A. fumigatus* is powdery, a large number of its spores readily disperse in the air to become a cause of infection. This species is not frequently isolated from the air. However, it is widely distributed in various indoor environments including hospitals, schools, houses, and the like. Such wide distribution seems to be a cause of infection[4,5,8,9,10]. Invasive pulmonary aspergillosis is highly fatal to susceptible patients undergoing immuinosuppressive therapy. Therefore, *A. fumigatus* is considered to be one of the most detrimental species in the hospitals[11].

The case of *Stachybotrys chartrum* in a flood disaster in the U.S. may be mentioned as a case of damage to human health by environmental mycotoxin. Mycotoxin produced by inhaled *S. chartrum* was pointed out as a possible cause of "idiopathic pulmonary alveolar hemorrhage in infants", which developed in infants[12].

There have been many reports on mycotoxins produced by fungi in food, which may be orally ingested. It is not known how fungi producing mycotoxins differ in influence on human health from those not producing mycotoxins when they are brought into the human body via the air. We experienced a case of a massive isolation of *A. ochraceus*, high in OTA producing capability, from the indoor air of a house and reported the development of chronic allergic symptoms, such as rhinitis, in three family members (a couple and their daughter) of the house[13]. We also reported a case of massive isolation of *A. fumigatus*, the causal fungus of severe pulmonary aspergillosis, from the air in a house in which a patient contracting the disease lived[14]. From such data, we infers that health damage due to the continuous inhalation to mycotoxin-producing fungi is greater than that due to the inhalation to non-producers.

Hence, we chose strains of *A. ochraceus* and *A. fumigatus* from the collection of isolates taken from the air of houses and public facilities examine their mycotoxin production level by liquid chromatography-tandem mass spectroscopy (LC/MS/MS). A number of strains were confirmed to produce mycotoxins and some were found very high in mycotoxin production capability. The results are described in this report.

Materials and Methods

Fungal Strains Test samples were 23 strains of *A. ochraceus* and 27 strains of *A. fumigatus*, which were obtained from the air of houses and public facilities. The isolates had been collected from the air with an SAS Super 100R Air Sampler (International Pbi) and Dicloran 18% glycerol agar plate (DG18). Both *Aspergillus* species were identified on the basis of their macro- and microscopic characteristics after subculturing on Czapek agar (Cz) according to a identification key[15,16]. As a further identification method, all samples of *A. fumigatus* cultured at 50°C, it was confirmed that the colonies[17].

Samples of *A. ochraceus* for mycotoxin analysis were 23 strains in total: eight strains from Tokyo, five strains from Kanagawa Prefecture, five strains from Chiba Prefecture, one strain from Saitama Prefecture, one strain from Ibaraki Prefecture, one strain from Shizuoka Prefecture, and two strains from Nagano Prefecture. Samples of *A. fumigatus* for mycotoxin analysis were 27 strains in total: 17 strains from Tokyo, three strains from Kanagawa Prefecture, four strains from Chiba Prefecture, one strain from Saitama Prefecture, one strain

from Shizuoka Prefecture, and one strain from Nagasaki Prefecture. Strain numbers, the years and places of isolation, and the classification of isolates in detail as well as results are given Tables 1 and 2.

Analysis of mycotoxins *A. ochraceus* strains were analyzed for ochratoxin A and ochratoxin B (OTA and OTB, hereafter). *A. fumigatus* strains were analyzed for gliotoxin and verruculogen.

A 50-mL Erlenmeyer flask to which 5 grams of barley grains, four milliliters of tap water, and one milliliter of 1% peptone water had been added was autoclaved. The rice and wheat grains were inoculated with *A. fumigatus* or *A. ochraceus* to be incubated at 20 ℃ for 10 days. To the incubated rice and wheat grains, 20 milliliters of ethyl acetate was added. The grains were then crushed with a medical spoon, left standing still for three hours, and filtered through a liquid phase separation filter. The filtrate was concentrated and dried at 70 ℃ using an evaporator. The resulting solid was dissolved in two milliliters of ethanol and served as the extract.

The extract, 0.2 mL, was transferred to a graduated test tube and diluted 13-fold with 50% methanol to prepare a test solution. Preliminary measurements were taken with an HPLC (Hewlet Packard 1090 HPLC DAD, 1046 FLD) with a diode array detector and a fluorescent detector in tandem. Samples of high concentrations were diluted with 20% methanol as required to take measurements with LC/MS/MS (Waters Alliance 2695 HPLC, Applied BioSystems API 3000). The column used for the measurement of OTA and OTB was

Table 1. Amount of ochratoxin A and ochratoxinB produced by *A. ochraceus.*

	Strain No.	Date	Origin	OTA (μg*)	OTB (μg*)
1	IFM 55911	2003 June	Karuizawa, Villa, Bath	3,200	<LOQ**
2	IFM 55912	2003 June	Karuizawa, Villa, Japanese-style room	4,300	<LOQ
3	IFM 59384	2004 May	Yokosuka, Detached house, Bedroom	4,300	<LOQ
4	IFM 59385	2004 May	Yokosuka, Detached house, Lavatory	1,800	<LOQ
5	IFM 59386	2004 May	Yokosuka, Detached house, Under floor	1,900	<LOQ
6	IFM 55625	2004 May	Yokosuka, Detached house, Kitchen	1,200	<LOQ
7	IFM 59390	2005 Mar.	Chiba, Detached house, Living room	1,100	<LOQ
8	IFM 59392	2005 May	Kodaira, Detached house, Japanese-style room	5,600	<LOQ
9	IFM 59393	2005 May	Kodaira, Detached house, Japanese-style room	210	34
10	IFM 59453	2005 Dec.	Katsushika, Apartment, Living room	2,100	<LOQ
11	IFM 59457	2006 July	Kodaira, Detached house, Living room	1,100	<LOQ
12	IFM 60772	2006 Sep.	Ageo, Detached house, Veranda	1,700	160
13	IFM 60773	2006 Nov.	Kodaira, Detached house, Veranda	3,300	<LOQ
14	IFM 56352	2007 Nov.	Ito, Art museum, Exhibition room	260	16
15	IFM 59460	2008 July	Nakano, Nursery school	1,500	<LOQ
16	IFM 59461	2008 Aug.	Kashiwa, Detached house, Japanese-style room	1,100	45
17	IFM 59462	2008 Sep.	Kashiwa, Detached house, Japanese-style room	1,100	15
18	IFM 59463	2008 Sep.	Yokohama, Apartment, Living room	1,500	52
19	IFM 59469	2009 Apr.	Funabashi, Apartment, Living room	670	<LOQ
20	IFM 59471	2009 July	Funabashi, Apartment, Living room	2,600	<LOQ
21	IFM 59464	2009 Aug.	Moriya, Detached house, Living room	6,100	<LOQ
22	IFM 59467	2009 Aug.	Kodaira, Detached house, Veranda	6,700	<LOQ
23	IFM 59470	2009 Aug.	Kodaira, Detached house, Garden	1,100	16

*production on 5 g of substrate. (single determination)

**limit of quantification, 0.1 μg corresponds to 0.011 mg/L as LC/MS/MS test soln.

Table 2. Amount of gliotoxin and verruculogen produced by *A. fumigatus.*

	Strain No.	Date	Origin	Gliotoxin (μg*)	Verruculogen (μg*)
1	IFM 60745	2005 July	Nagasaki, Detached house, Japanese-style room	<LOQ**	380
2	IFM 60746	2005 Nov.	Ota, Apartment, Japanese-style room	<LOQ	460
3	IFM 60747	2005 Nov.	Ota, Apartment, Japanese-style room	<LOQ	<LOQ**
4	IFM 60748	2005 Nov.	Ota, Apartment, Veranda	<LOQ	990
5	IFM 60749	2005 Nov.	Ota, Apartment, Veranda	<LOQ	480
6	IFM 60750	2005 Nov.	Kodaira, Detached house, Japanese-style room	36	620
7	IFM 60751	2005 Nov.	Kodaira, Detached house, Japanese-style room	<LOQ	1,200
8	IFM 60752	2005 Nov.	Kodaira, Detached house, Japanese-style room	<LOQ	360
9	IFM 60753	2005 Dec.	Kodaira, Detached house, Living room	<LOQ	1,300
10	IFM 60754	2005 Dec.	Kodaira, Detached house, Living room	46	560
11	IFM 60755	2005 Dec.	Kodaira, Detached house, Living room	<LOQ	400
12	IFM 60756	2005 Dec.	Kodaira, Detached house, Japanese-style room	74	<LOQ
13	IFM 60757	2005 Dec.	Kodaira, Detached house, Japanese-style room	<LOQ	720
14	IFM 60758	2005 Dec.	Kodaira, Detached house, Japanese-style room	31	820
15	IFM 60759	2005 Dec.	Kodaira, Detached house, Veranda	<LOQ	1,200
16	IFM 60760	2006 May	Ageo, Detached house, Living room	<LOQ	2,700
17	IFM 60761	2006 June	Adachi, Apartment, Living room	<LOQ	2,000
18	IFM 60762	2006 July	Bunkyo, Nursing home, Air conditioning	<LOQ	400
19	IFM 60763	2008 July	Yokohama, Apartment, Living room	<LOQ	1,100
20	IFM 60764	2008 July	Sagamihara, Detached house, Living room	<LOQ	1,300
21	IFM 60765	2009 Apr.	Funabashi, Apartment, Living room	<LOQ	<LOQ
22	IFM 60766	2009 May	Funabashi, Apartment, Living room	<LOQ	350
23	IFM 60767	2009 July	Funabashi, Apartment, Living room	<LOQ	1,500
24	IFM 60768	2009 Aug.	Yokohama, Apartment, Living room	<LOQ	1,100
25	IFM 60769	2009 Aug.	Setagaya, Apartment, Living room	<LOQ	1,200
26	IFM 60770	2009 Sep.	Funabashi, Apartment, Living room	<LOQ	520
27	IFM 60771	2009 Sep.	Ito, Art museum, Exhibition room	<LOQ	1,200

*production on 5 g of substrate. (single determination)

**limit of quantification, 0.1μg corresponds to 0.011 mg/L as LC/MS/MS test soln.

Inertsil ODS-3V (GL Sciences, 5 μm, 2.1 mm I.D.×150 mm), which was gradient-eluted with acetonitrile and 5 mM ammononium acetate buffer; Symmetry C18 (Waters, 5 μm, 2.1 mm I. D.×150 mm) was used for gliotoxin and verruculogen to be gradient-eluted with acetonitrile and water.

For toxin detection, OTA and OTB were ionized in an ESI negative mode with the former measured at precursor ion m/z 402.1 and product ion m/z 357.9 and 166.9 and the latter at precursor ion m/z 368.1 and product ion m/z 324.0 and 280.0. Gliotoxin and verruculogen were ionized in an ESI positive mode with the former measured at precursor ion m/z 534.4 and product ion m/z 392.0 and 435.0 and the latter at precursor ion m/z 326.8 and a product ion m/z 263.0 and 244.6. Limits of detection and limits of quantification were around 0.003 mg/L and 0.011 mg/L respectively.

Results and Discussion

Table 1 shows the amounts of OTA and OTB the tested *A. ochraceus* strains produced with barley grains. Table 2 shows the amounts of gliotoxin and verruculogen *A. fumigatus* produced. It should be noted that most

of the strains produced large amounts of mycotoxins.

A. ochraceus strains tested in this study produced 2367 μg* of OTA on an average. OTB was produced by seven of 23 strains. The ratio of OTB producers was lower (30.4%) than that of OTA producers. The result revealed that many of airborne *A. ochraceus* strains were OTA producers. As mentioned previously, there were cases in which *A. ochraceus* grew massively. Therefore, it is necessary to be careful when monitoring fungi in indoor environment.

A. fumigatus has been reported to produce various mycotoxins in addition to gliotoxin and verruculogen that were analyzed in this study, such as fumigallin, fumitoxin, fumigaclavines, fumigatin, fumitremorgins, monomethylsulochrin, helvolic acid, pseurotinx, phripyropens, methyl-sulochrink, traypacidin, and tryptoquivaline[18]. Genera *Aspergillus*, *Penicillium*, *Gliocladium*, *Thermoascus*, and *Candida* produce gliotoxin, which has been found to possess various biological activities[19]. Genera *Aspergillus* and *Penicillium* produce verruculogen, which is a low-molecule mycotoxin and known to show neurotoxicity in mouse and rat[20]. Gliotoxin was produced by 14.8% of strains tested in this study, indicating most of the strains did not produce gliotoxin. In contrast, 88.9% of the strains produced verruculogen (an average of 847 μg*), confirming that many of *A. fumigatus* strains produce verruculogen. There is a report on verruculogen production by indoor airborne strains and clinical isolates obtained in a hospital in Zagreb, Croatia[20]. Most of the data are in agreement with those of this study, although different analytical methods were used.

A. ochraceus and *A. fumigatus* are common fungal species in the air of houses and can conceivably become dominant species sometimes. Therefore, it cannot be ruled out that residents may inhale them continuously and be exposed to mycotoxins the fungal species produce. This study focused on typical mycotoxins *A. ochraceus* and *A. fumigatus* produce. *S. chartrum* besides the two species has been experimentally proven to cause "pulmonary hypertension symptoms" in mouse when inhaled continuously, which are different from poisoning and allergy[12] caused by mycotoxin this fungus produces. From such a viewpoint, it is desirable to conduct similar surveys on other mycotoxins in living environment, particularly in the air.

We plans to accumulate analytical data of mycotoxins on indoor airborne isolates and survey data on health conditions of residents to find health damage caused by the continuous inhalation of fungi that produce mycotoxins.

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空気中から分離された *Aspergillus ochraceus* および *Aspergillus fumigatus* のマイコトキシン産生量

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日本国内の一般住宅や公共施設の浮遊真菌の調査を行った。この調査によって分離された *Aspergillus* 属の菌株を約 500 株保存している。これら保存菌株の中から，2003 年～2009 年に空気中から分離された *Aspergillus ochraceus* と *Aspergillus fumigatus* を対象に，LC/MS/MS を用いて，マイコトキシン産生量を分析した。*A. ochraceus* は ochratoxin A と ochratoxin B の産生量について，*A. fumigatus* は gliotoxin と verruculogen の産生量について，それぞれ分析した。この結果，分析した株の多くでマイコトキシン産生能が確認された。特に，空気中に浮遊する *A. ochraceus* の中には，ochratoxin A の産生量が比較的高い菌胞子が浮遊していることが明らかになった。

キーワード：Aspergillus ochraceus, Aspergillus fumigatus, ochratoxin A, ochratoxin B, gliotoxin, verruculogen, 空中浮遊真菌