Studies on the Population of Toxigenic Fungi in Foodstuffs

IV. Aflatoxin Producing Fungi Isolated from Foodstuffs in Japan

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Hiroshi KURATA, Hiroya TANABE*, Kenzo KANOTA*, Shun-ichi UDAGAWA
and Masakatsu ICHINOE

(Laboratory of Mycology; Laboratory of Food Chemistry*, National Institute
of Hygienic Sciences, 203, 2 chome, Tamagawa-Yoga, Setagaya-ku, Tokyo, Japan)

Fungal isolates from rice and flours were tested to determine if aflatoxin producing fungi are present in Japan. One hundred and fifteen strains, including 21 strains of the *Aspergillus flavus* group, were tested. Fifty one of 115 strains were isolated from rice samples and the others from several kinds of flours including wheat, red bean, and soybean. The isolates were selected from a total of 1300 on the basis of frequency of isolation of species except that all *Aspergillus flavus* isolates were tested. In primary screening tests, the cultures were grown in shake culture for 96 hours in modified Czapek liquid medium. The presence of aflatoxins were determined by thin-layer chromatography of chloroform extract of the broth. Eleven isolates including 5 belonging to the *Aspergillus flavus* group appeared to produce aflatoxin B₁ alone or B₁ and B₂. Further large scale tests indicated that strains of *Aspergillus flavus* produced compounds which showed the same thin layer chromatographic behaviours as authentic aflatoxin B₁. Production of aflatoxin B₁ was confirmed by the trifluoroacetylation procedure of Andrellos and Reids (1964), and by measurement of ultraviolet and infrared spectra. Oral administration to rats of extract of one strain of *Aspergillus flavus* (WF-3-8) caused histopathological change to the livers accompanied with bile duct hyperplasia similar to that observed in the liver of rats fed toxic peanut meal.

This is the first report of aflatoxin producing fungi isolated from foodstuffs in Japan. No aflatoxin producing strains were found among those isolated from rice samples.

Introduction

During the past several years, a great deal of interest has been aroused by the discovery of the mycotoxins^{1)~4)}, patulin⁵⁾, islanditoxin⁶⁾⁷⁾, aflatoxin⁸⁾⁹⁾ and Ochratoxin¹⁰⁾¹¹⁾. In 1960, a fungal metabolite with carcinogenic activity, was first found in Japan by Tsunoda¹²⁾ and Uruguchi et al.¹³⁾ This toxin, a chlorine-containing cyclic peptide elaborated by *Penicillium islandicum* growing on imported rice grains was named islanditoxin. Shortly thereafter, aflatoxin, coumarin-like compounds, was found in

Brazilian peanut meal infested by some strains of *Aspergillus flavus*⁸⁾⁹⁾¹⁴⁾¹⁵⁾¹⁶⁾. It caused a high incidence of hepatomas and renal damage when fed to rats¹⁴⁾¹⁷⁾¹⁸⁾. At least five aflatoxins are known to exist and structures of B₁, B₂, G₁, G₂, and M₁ have been determined⁴⁾¹⁹⁾²⁰⁾. Furthermore, several workers^{21)~25)} have reported the aflatoxin producing fungi associated with various kinds of foodstuffs, and nine species of fungi have been reported to produce aflatoxins: *Aspergillus flavus*, *A. parasiticus*, *A. niger*, *A. ruber*, *A. wentii*, *Penicillium citrinum*, *P. cyclopium*, *P. puberulum*, *P. frequentans*, and *P. variable*. In view of this evidence, it seemed reasonable to assume that some species of fungi originating in Japanese foodstuffs may produce aflatoxins. From the results of aflatoxin assays in 1966, Yokotsuka²⁶⁾ and Sasaki²⁷⁾ stated that there were no aflatoxin producing fungi among about one hundred strains of the *Aspergillus flavus-oryzae* group being used by the fermentation

* 倉田 浩, 田辺弘也, 叶多謙蔵, 宇田川俊一, 一戸正勝 (国立衛生試験所)

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industry in Japan. Similar results were also obtained by Miyaki et al.⁽²⁸⁾ and Matsuura⁽²⁹⁾. The present study was made to determine if aflatoxin producing fungi were present in Japanese foodstuffs.

Materials and Methods

Strains of fungi tested. The mycoflora of 319 samples of foodstuffs such as wheat flour, redbean flour, soybean flour, kidneybean flour, buckwheat flour, rice grain and rice flour were examined in previous studies⁽³⁰⁾⁽³¹⁾ and about 1300 colonies of

Table 1. Number of Samples of Foodstuffs, and Total Number of Fungal Colonies from Each Sample

Kinds of samples	No. of samples examined	Total No. of colonies isolated
Rice grain	219	835
Wheat flour	50	288
Rice flour	15	83
Soybean flour	15	32
Red-bean flour	6	20
Kidneybean flour	4	4
Buckwheat flour	5	25
Potato starch	5	13
Total	319	1,300

fungi were isolated on Peptone-glucose plates containing chloramphenicol (Table 1). Of these colonies, 115 isolates were selected in an accordance with the frequency of their occurrence and assayed for the ability to produce aflatoxins on culture media. All isolates were transferred to slants of PDA and PCA agar (Potato-Carrot agar) and maintained in sealed tubes under refrigerated storage. These fungi were mostly *Penicillium* and *Aspergillus* species. The number and kind of fungi tested are listed in Table 2.

Culture media. Liquid medium was used in the primary screening test⁽³²⁾, while solid medium (crushed wheat) was used as substrate in the secondary confirmatory test. The constituents of the liquid medium were as follows: Glucose 50.0g, casamino acid (Difco) 6.35 g, KH_2PO_4 10.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2.0 g, $\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O}$ 10.0 mg, $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.11 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 17.6 mg. The liquid cultures were shaken in Sakaguchi flasks (500 ml) for 96 hours at 30°C. Erlenmyer flasks (5,000 ml) containing 0.5 kg of roasted and crushed wheat mixed with 300 ml of tap water were prepared for the solid medium. This medium was autoclaved for 20 min. at 121°C. After inoculation with test fungi, the flasks were incubated for two weeks at 30°C. During incubation, the flasks were agitated by hand at least once per day for some minutes to prevent the substrate from forming a compact mass. Each flask was inoculated with conidia adhering to a wet

Table 2. List of Fungi Examined

<i>Aspergillus flavus</i>	21	<i>Penicillium cyclopium</i>	5
" <i>versicolor</i>	10	" <i>viridicatum</i>	5
" <i>candidus</i>	5	" <i>ochraceum</i>	3
" <i>repens</i>	5	" <i>spinulosum</i>	3
" <i>ruber</i>	5	" <i>citrinum</i>	2
" <i>ochraceus</i>	4	" <i>roqueforti</i>	2
" <i>clavatus</i>	3	" <i>frequentans</i>	1
" <i>niger</i>	3	" <i>funiculosum</i>	1
" <i>oryzae</i>	3	" <i>islandicum</i>	1
" <i>amstelodami</i>	2	" <i>notatum</i>	1
" <i>fumigatus</i>	2	" <i>puberulum</i>	1
" <i>terreus</i>	2	" <i>wortmanii</i>	1
" <i>nidulans</i>	1	" <i>sp.</i>	1
" <i>sydowi</i>	1		
" <i>ustus</i>	1	<i>Chaetomium globosum</i>	1
<i>Cladosporium cladosporioides</i>	5	<i>Phoma</i> spp.	2
<i>Helminthosporium oryzae</i>	4	<i>Alternaria tenuis</i>	1
<i>Fusarium</i> spp.	3	<i>Curvularia lunata</i>	1
<i>Trichoconis padwickii</i>	2	<i>Piricularia oryzae</i>	1
		Total	115

inoculating loop from a 10~14 day-old PDA slant culture.

Aflatoxin assay for screening test. Isolation of Aflatoxins: All the liquid culture (150 ml) was shaken with 3 portions of chloroform (150, 100, 100 ml). All chloroform extracts were combined, dried with anhydrous sodium sulfate, and concentrated *in vacuo* almost to dryness using a modified Kuderna-Danish evaporator. The residue was dissolved in a small amount of chloroform and ten times its volume of cold *n*-hexane was added. Precipitates were removed by filtration, and washed with cold *n*-hexane. For application to thin layer chromatography, the precipitates were dissolved in 1 ml of chloroform.

Thin layer chromatography^{(38)~(39)}: Each of the samples and standard solutions in chloroform were spotted side by side on a thin layer chromatographic plate of 0.25 mm thick, prepared with silica gel G according to Stahl and activated at 105°C for 30 min. After development with methanol:chloroform (2:98 v/v) the spots were compared under ultraviolet light.

Identification of aflatoxin B₁. Purification of aflatoxin B₁^{(33) (38) (39)}: To free the B₁ component from the other toxins and extraneous matter, crude precipitates obtained in the same method as described above were separated by preparatory thin layer chromatography on silica gel, using methanol:chloroform (2:98 v/v) as the developer. The spots corresponding to aflatoxin B₁ were scraped from the plate into a suitable container, and eluted with chloroform. After removal of the silica gel by filtration and of chloroform by evaporation, the residual material was tested as follows. Confirmatory test for aflatoxin B₁: Trifluoroacetylation method^{(33) (40)} by Andrellos and Reids⁽³³⁾ was slightly modified. Chloroform solutions of the purified sample and standard aflatoxin B₁ were spotted side by side on the line of origin of a thin layer chromatographic plate. Each of these spots was sprayed with several μ l of trifluoroacetic acid and

then the plate was developed with methanol:chloroform (2:98 v/v). The spot of the reaction product of the sample was compared under ultraviolet light with that of the treated standard for identification. Absorption spectrographic identification of aflatoxin B₁: UV absorption spectra of methanol solutions of the purified samples and their molar absorptivities were compared with those of the standard. Infrared absorption spectra of the purified sample taken by potassium bromide tablet method were also compared with those of the standard.

Animal toxicity test. Winster strain rats, 120~150 g, male, were given a single administration of crude and pure crystalline toxins obtained from the WF-3-8 strain. Both compounds were dissolved in propylene glycol. Either 2 ml of propylene glycol solution containing 0.942 mg of pure crystalline or 3.232 mg of crude aflatoxin were given into the stomach through a metal cannula. A control animal were given a similar volume of the solvent. All surviving animals were killed 7-days after the start of the examination. Animals killed or dying were autopsied and tissues fixed in 10 per cent formal saline. Paraffin section prepared in the usual manner were stained with haematoxyline and eosin and in some cases with P.A.S.

Results and Discussion

Liquid cultures of 11 strains in the first screening test were suspected to contain aflatoxins. Five of 21 strains of *Aspergillus flavus* and one of 10 strains of *Aspergillus versicolor* were appeared to produce aflatoxin B₁ and B₂. Each one of 5 strains of *Aspergillus candidus*, 2 strains of *Aspergillus fumigatus*, 3 strains of *Fusarium* species and 5 strains of *Cladosporium cladosporioides* were suspected to produce aflatoxin B₁ alone (Table 3). These 11 strains were massively cultured using solid media for further confirmatory tests. None of the strains produced aflatoxin G₁ or G₂-like substances.

Table 3. Results of the Primary Screening Test for Aflatoxins Producing Strains

Fungal species	No. of strains tested	No. of strains producing aflatoxin-like compds.	Aflatoxins
<i>Aspergillus flavus</i>	21	5	B ₁ , B ₂
" <i>versicolor</i>	10	1	B ₁ , B ₂ ?
" <i>candidus</i>	5	1	B ₁
" <i>ruber</i>	5	1	B ₁
" <i>fumigatus</i>	2	1	B ₁
<i>Cladosporium cladosporioides</i>	5	1	B ₁
<i>Fusarium</i> spp.	3	1	B ₁

The B₁-like substances obtained by extraction of 14 day old cultures of solid media of the 11 strains mentioned above were tested by a second confirmatory chemical test and absorption spectrographic identification. In the confirmatory tests, the fluorescent aflatoxin B₁-like substance obtained from the solid cultures of 2 strains of *Aspergillus flavus* from flours of wheat and red bean respectively were proved to be identical with authentic aflatoxin B₁. The substance produced by the two strains behaved similar to authentic aflatoxin B₁ not only in thin layer chromatography of themselves but also in that of trifluoroacetylation product (Fig. 1).

Kiesel Gel G by Stahl, 0.25 mm, activated at 105° for 30 min. Developer consists of 98 parts of chloroform and 2 parts of methanol.

Fig. 1. TLC behavior of chloroform extracts of products of *Asp. flavus* which were suspected to produce Aflatoxins

The UV spectra of the methanol solutions of each of these two substances showed absorptions at 223, 240 (shoulder), 265 and 362 mμ, the molar absorptivity value of each absorption were 2.16×10^4 , 3.10×10^4 , 1.17×10^4 and 2.18×10^4 , respectively. These were well in accord with authentic aflatoxin B₁.

The IR spectra of the two substances also completely agreed with that of authentic aflatoxin B₁, wave number of characteristic absorptions being 1760 (very intense), 1684 (weak), 1632, 1598 and 1562 (Fig. 2~3).

The m.p. of these two substances were around 265~267°C, however, this seemed to be due to slight impurities of the samples and judging from

Fig. 2. UV absorption spectra of the compounds which show same Rf value as authentic Aflatoxin B₁

Fig. 3. IR absorption spectra of the compounds which show same Rf value as authentic Aflatoxin B₁

the data mentioned above, these two substances were considered to be identical with aflatoxin B₁.

Even if we were not successful in obtaining pure aflatoxin B₂ due to low concentration, thin layer chromatographic data and the presence of aflatoxin B₁ strongly suggest that B₂ is produced by both of the strains of *Aspergillus flavus* originated from flours of wheat and red bean.

The acute toxicity test using crude and pure compounds extracted from strain WF-3-8 was carried out on four rats. One rat receiving the pure compound died at the 2nd day. The remainder were killed and autopsied at the 7th day after administration. Histopathological examination of the organs of the rats was carried out by routine procedures and the findings were as follows:

i) Rats administrated crude compound. The

liver showed widespread necrosis of hepatic cells, namely, severe necrosis of cells in the central regions and marked degeneration in the middle zones of the lobuli were observed. Sinusoids were dilated and reticular network in the central area showed lytic change. Acidophilic bodies were occasionally found in the middle zone of the lobuli. Proliferation of Kupffer's cells was remarkable, however, no inflammatory cell infiltration in and around the hepatic lobuli was revealed. No bile duct proliferation was recognized in the periportal area.

Compact or dark cells were observed scattered in the adrenal cortex. Edematous change of tubular epithelium and cystic dilatation of tubuli of lower nephrons were noteworthy findings in the kidney. Myocardial petechial hemorrhage and edema of the other organs were noticed.

ii) Rats administered pure compound. The liver indicated subacute hepatic injury. The peripheral zone of hepatic lobuli was almost completely occupied by diffuse proliferation of pseudobile ducts. Acidophilic bodies were scattered in that lesion. Slight fibrosis was noticed in the periportal sheath, however, neither bile duct proliferation nor reactive cell infiltration were observed.

A marked cystic dilatation of tubuli of lower nephrons was the noteworthy finding in the kidney.

As Allcroft et al.⁽¹⁴⁾⁽¹⁹⁾ and Butler⁽¹⁷⁾⁽¹⁸⁾ have reported, reduction in growth and liver size, and the extent of bile duct proliferation are the criteria of toxicity of aflatoxin. It could be assumed, therefore, that the compounds from WF-3-8 has a property of aflatoxigenicity. Further work on the toxicity of compounds from AF-4-4 is now being pursued.

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