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**Production of Aflatoxins and Aflatoxicols by *Aspergillus flavus* and
Aspergillus parasiticus and Metabolism of Aflatoxin B₁ by
Aflatoxin-non-producing *Aspergillus flavus***

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The production of aflatoxin (AF) groups (B₁, B₂, G₁, G₂, M₁, M₂, P₁ and Q₁) and aflatoxicol (AFL) groups (A and B) by AF-producing *Aspergillus flavus* and *A. parasiticus* isolated from various foods, and the metabolism of AF B₁ to AF M₁, AF P₁, AF Q₁, AFL-A and AFL-B by AF-non-producing *A. flavus* also isolated from various foods, were studied. A simple and rapid method for the analysis of the 10 kinds of toxins was developed.

Toxins produced in culture were extracted with chloroform, separated into AF and AFL fractions by a silica gel cartridge column, then assayed by high performance liquid chromatography (HPLC).

AF M₁, AF M₂, AFL-A and AFL-B were detected in addition to AF B₁ and AF B₂ in nearly all of the cultures inoculated with AF-producing *A. flavus*. The same groups were detected in addition to AF B₁, AF B₂, AF G₁ and AF G₂ in the cultures inoculated with *A. parasiticus*. The time courses of AF M₁ and AF M₂ production were almost the same as those of AF B₁, AF B₂, AF G₁ and AF G₂. AFLs tended to appear after AFs had accumulated in the cultures. AF P₁ and AF Q₁ were not detected at all.

AF B₁ added to the cultures was metabolized by all strains of non-AF-producing *A. flavus*. AFL-A and AFL-B were the common metabolites in all of the cultures. However, no strains which converted AF B₁ to AF M₁, AF P₁ and AF Q₁ were observed.

Keywords — mycotoxin; aflatoxin; aflatoxicol; *Aspergillus flavus*; *Aspergillus parasiticus*; HPLC; fluorescence detector; silica gel cartridge column; MS

Introduction

Aflatoxins are a group of structurally related toxic bisfuranocoumarins produced as secondary metabolites by certain strains of *Aspergillus flavus* and *A. parasiticus*. Typical aflatoxins produced by these species are B₁, B₂, G₁, and G₂, frequently found as natural contaminants of both human and animal food-stuffs. These species are also well known¹⁾ to produce some aflatoxin (AF)-related com-

pounds such as M₁, M₂, B_{2a}, G_{2a}, Parasiticol, etc. Natural contamination by these toxins, however, is not generally found. Among these metabolites, AF B₁, which is produced most abundantly by these *Aspergillus* species is the most highly carcinogenic, and it has been the subject of numerous studies. In AF B₁ degradation or metabolism studies, several derivatives have been identified. AF M₁, AF P₁, AF Q₁ and aflatoxicol (AFL)-A are well known¹⁾ as metabolites in animals. However, it is

known that AF B₁ is also metabolized by many species of fungi,²⁾ in which AF B_{2a},^{3,4)} AFL-A⁵⁻⁸⁾ and AFL-B⁵⁻⁸⁾ have been identified as metabolites.

The authors found that maize naturally contaminated with high concentrations with AF B₁ and AF B₂ was simultaneously contaminated with AF M₁, AF M₂, AFL-A and AFL-B,⁹⁾ and found that *A. flavus* isolated from maize contaminated with AFs and AFLs produced AF M₁, AFL-A and AFL-B in addition to AF B₁ and AF B₂.¹⁰⁾ Furthermore, we reported that several other fungi (*A. niger*, *Eurotium herbariorum*, *Rhizopus* sp., etc.) isolated from the same maize had fairly strong ability to convert AF B₁ to AFL.⁸⁾

If AF B₁-producing *A. flavus* and *A. parasiticus* have the ability to produce various AF B₁ derivatives or to convert AF B₁ to AF B₁ derivatives, these AF B₁-producing strains should be the first suspects as strains causing the contamination of the AF B₁ derivatives. However, little is known about the production abilities of AF B₁ derivatives or the conversion abilities of AF B₁ to AF B₁ derivatives in AF B₁-producing *A. flavus* and *A. parasiticus*.

This report deals with the production of the well characterized metabolites of AF B₁, i.e., AF P₁, AF Q₁, AFL-A and AFL-B, as well as those of AF B₁, AF B₂, AF G₁, AF G₂, AF M₁ and AF M₂ of *A. flavus* and *A. parasiticus* isolated from various commercial foods. The conversion of AF B₁ to the above mentioned AF B₁ derivatives by AF-non-producing *A. flavus* is also described. Further, a simple method for isolation and determination of these 10 toxins by high performance liquid chromatography (HPLC) is given.

Materials and Methods

Organisms — The production of AFs and AFLs was investigated in AF-producing *A. flavus* (18 strains) and *A. parasiticus* (3 strains) isolated from commercial foods. The metabolism of AF B₁ was investigated in 3 strains of *A. flavus* which lost AF productivity at the subculture stage and 4

strains of *A. flavus* without AF productivity at the isolation stage.

Culture Medium and Reagents — Low-salt (SL) medium¹¹⁾ was used to study the production of AFs and AFLs by AF-producing *A. flavus* and *A. parasiticus* and the metabolism of AF B₁ by AF-non-producing *A. flavus*. AFL-A, AFL-B, AF B₁, AF B₂, AF G₁, AF G₂, AF M₁, AF M₂, AF P₁ and AF Q₁, were obtained from Makor Chemicals, Ltd. (Jerusalem, Israel). These toxins were used as standard without refining.

Inoculation and Incubation — All strains were inoculated onto potato dextrose agar (PDA) slants and cultivated for 7 days at 25 °C until well sporulated. The spores were harvested in 0.001% Tween 80 to a density of 10⁴ spores per ml. One hundred microliters of the spore suspension from AF-producing strains was inoculated onto 10 ml of SL medium, and the medium cultured for 5, 10, 15 and 20 days at 25 °C under stationary conditions. After incubation, the production of AF B₁, AF B₂, AF G₁, AF G₂, AF M₁, AF M₂, AF P₁, AF Q₁, AFL-A and AFL-B was investigated. To study the conversion of AF B₁ to AF M₁, AF P₁, AF Q₁, AFL-A and AFL-B, 100 µl of the spore suspension prepared from AF-non-producing *A. flavus* was inoculated onto 10 ml of SL culture medium containing AF B₁ (2000 ng/ml), and cultured under the same conditions described above. After the incubation, levels of AF B₁ metabolites were investigated.

Extraction of AFs and AFLs and Preparation of Test Solution — Five grams of sodium chloride and 100 ml of chloroform were added to the whole culture. The mixture was homogenized at 10000 rpm for 10 min and centrifuged to separate the chloroform layer. The chloroform layer was then dehydrated with anhydrous sodium sulfate and filtered through filter paper. The solvent was concentrated to approximately 5 ml under reduced pressure. For separation into AF and AFL groups, the concentrated solvent was applied to a Mega Bond Elut Silica gel column (Sorbent mass, 5 g; Analytichem International, Harbor City, CA, U.S.A.) previously washed with 20 ml of chloroform. The column was washed with 20 ml

of *n*-hexane, and then AFLs were eluted with 20 ml of ethylether. The remaining AFs were eluted with 40 ml of methanol–chloroform (3:97). The solvent was evaporated from the eluates, and residues were dissolved in an appropriate amount of chloroform for HPLC.

HPLC Conditions—The HPLC system was composed of a 880-PU pump (Jasco), a 470-type scanning fluorescence detector (Waters Associates, Milford, Massachusetts, U.S.A.), and a C-R6A data processing unit (Shimadzu). HPLC conditions for the determination of the AF group, *i.e.*, B₁, B₂, G₁, G₂, M₁, M₂, P₁ and Q₁ were as follows: column, Finepak SIL-5 (5 μ m, 25 cm $\times$ 4.6 mm I.D., Jasco); mobile phase, toluene–ethylacetate–formic acid–methanol (700:70:15:20); flow rate, 1.0 ml/min; detection, fluorescence (excitation 365 nm, emission 425 nm). HPLC conditions for the determination of AFL-A and AFL-B were as follows: column, Finepak SIL-5 (5 μ m, 25 cm $\times$ 4.6 mm I.D.); mobile phase, chloroform–methanol (99:1); flow rate, 1.0 ml/min; and detection, fluorescence (excitation 330 nm, emission 390 nm). Chloroform (Kanto Chemical CO.) containing amilene as a stabilizer was used as the main component of the HPLC mobile phase.

Confirmation of AFs and AFLs—AFs were confirmed by the excitation and emission spectra of the HPLC peaks. The spectra were compared with those of standard substances. AFLs were confirmed by mass spectrometry (MS). AFL samples for HPLC were further divided into AFL-A and AFL-B fractions by normal phase HPLC with chloroform–methanol (100:0.5) as the mobile phase. The respective fractions were concentrated to 50–100 μ g/ml AFL in chloroform, and 1 μ l of the fraction was applied to MS by direct introduction (DI).

MS Conditions—Mass spectrometer, TSQ 70 (Finigan MAT, San Jose, CA, U.S.A.); ionization mode, electron ionization (EI); ion source temp., 150°C; ionization voltage, 70eV; emission current, 700 μ A; detector voltage, 1000V.

Results and Discussion

1. Analytical Conditions

For analysis of metabolites in the culture, 8 types of AF, *i.e.*, B₁, B₂, G₁, G₂, M₁, M₂, P₁ and Q₁, and 2 types of AFL, *i.e.*, AFL-A and AFL-B, were selected as the subjects of investigation. Optimum conditions for extraction of the toxins from cultures and for their isolation by HPLC were studied.

1) Conditions for Extraction of AFs and AFLs—Ten types of toxin were recovered by chloroform extraction¹⁰⁾ from the cultures. Five grams of sodium chloride and 100 ml of chloroform were added to 10 ml of SL culture medium containing 5 ppm of each toxin. The mixture was homogenized at 10000 rpm for 10 min, resulting in over 95% recoveries of AFs and AFLs. Therefore, chloroform was considered the most suitable extraction solvent.

2) HPLC Conditions for AFs and AFLs—AFs and AFLs were simultaneously determined with a HPLC fluorescence detector. It is known¹²⁾ that AF B₁ and AF G₁ in reverse phase solvents and AF B₁ and AF B₂ in normal phase solvents consisting mainly of *n*-hexane or chloroform emit very weak fluorescence. Therefore, in general, AF B₁ and AF G₁ were converted to the hemiacetals, AF B_{2a} and AF G_{2a}, in order to intensify their fluorescence in reverse phase solvents, and were measured by reverse phase HPLC with a fluorescence detector.^{13–15)} This method is only applicable to some AFs, however, being unsuitable for the simultaneous analysis of various toxins. Manabe *et al.*¹⁶⁾ found that the fluorescence intensities of AF B₁ and AF B₂ were increased by the addition of acids to the mobile phase, and performed a high sensitivity analysis of AF B₁, AF B₂, AF G₁ and AF G₂ by fluorescence using a silica gel column and toluene–ethylacetate–formic acid–methanol as the mobile phase. We first determined whether or not simultaneous determination of the 10 AFs and AFLs would be feasible under

these conditions, and found that the fluorescence of AFs and AFLs was strong enough in this mobile phase to enable detection at high sensitivity. However, for the reciprocal separation of each toxin, it was necessary to modify the solvent composition in the mobile phase. In particular, the ethylacetate concentration in the mobile phase greatly influenced the separation of 8 AFs (Fig. 1). When the solvent composition of this mobile phase was 700:70:15:20, the 8 AFs were isolated in a short time (Fig. 2). However, AFL-A and AFL-B maintained the exact same retention time, being eluted under such conditions first (in about 8 minutes). Furthermore, it is also known that parts of AFL-A and AFL-B react with methanol in the presence of formic acid in the mobile phase. They appear as new peaks at a retention time of about 4 to 5

minutes. The retention time of these new peaks is shorter than those of AFL derivatives which react with ethanol. Cole *et al.*⁶⁾ reported that parts of AFL-A and AFL-B reacted with ethanol in the eluting solvent and were respectively converted to AFL-O-ethylether A and AFL-O-ethylether B during silica gel column chromatography. This is similar to the appearance of the new AFL derivatives in the present investigation. Therefore, it was considered that the new derivatives were AFL-O-methylether A and AFL-O-methylether B, respectively. Accordingly, AFL could not be assayed in this mobile phase. Assay of AFL-A and AFL-B was thus carried out under different conditions previously reported by the authors,⁸⁾ *i.e.*, using silica gel for the column and chloroform-methanol (99:1) as the mobile phase. Under these conditions, AFL-A and AFL-B were very well separated (Fig. 3). However, since the retention time of AF B₁ was very close to that of AFL-A, the complete isolation of AFL-A from AF B₁ was almost impossible. Thus, both the AFL and AF groups required fractionation prior to HPLC.

Fig. 1. Effect of the Volume Ratio of Ethylacetate on the Retention time of Aflatoxins

mobile phase; toluene-ethylacetate-formic acid-methanol (700:X:15:20).

—▲—: aflatoxin B₁, —△—: aflatoxin B₂, —■—: aflatoxin G₁,
—□—: aflatoxin G₂, —▼—: aflatoxin M₁, —▽—: aflatoxin M₂,
—●—: aflatoxin P₁, —○—: aflatoxin Q₁.

Fig. 2. High Performance Liquid Chromatogram of Aflatoxins

a: aflatoxin B₁ (20 ng), b: aflatoxin B₂ (10 ng), c: aflatoxin G₁ (10 ng), d: aflatoxin P₁ (20 ng), e: aflatoxin Q₁ (40 ng), f: aflatoxin G₂ (5 ng), g: aflatoxin M₁ (20 ng), h: aflatoxin M₂ (30 ng).

Fig. 3. High Performance Liquid Chromatogram of Aflatoxicols

a: aflatoxicol A (10 ng), b: aflatoxicol B (8 ng).

3) Pretreatment with Silica Gel Cartridge Column — We fractionated 2 AFLs and 6 AFs (B_1 , B_2 , G_1 , G_2 , M_1 and M_2) by silica gel column chromatography, and reported that because only AFL-A and AFL-B are eluted by ether, fractionation of AFLs and AFs is feasible.¹⁷⁾ We applied this method to the analysis of the 2 AFLs and the 8 AFs with the silica gel cartridge column. AFL retained in the column was eluted completely in 20 ml of ether, but AF was hardly eluted. AFs were eluted with a mixture of chloroform-methanol (9:1). AF B_1 and AF B_2 were eluted completely in 20 ml, while other AFs required 30 to 35 ml of solvent to be eluted completely. We thus selected 40 ml as the elution volume. With the cartridge column, the clean-up time was shortened notably and the amount of clean-up solvent was decreased markedly (1/5—1/10), compared with the previous method.¹⁷⁾

2. AF and AFL Productivity of AF-Producing *A. flavus* and *A. parasiticus*

The production of the AF group, *i.e.*, B_1 , B_2 , G_1 , G_2 , M_1 , M_2 , P_1 and Q_1 , as well as AFL-A and AFL-B by AF-producing *A. flavus* and *A. parasiticus* was examined. In most of the cultures inoculated with AF-producing *A. flavus*, AF B_1 , AF B_2 , AF M_1 , AF M_2 , AFL-A and AFL-B were detected. AF B_1 , AF B_2 , AF G_1 , AF G_2 , AF M_1 and AF M_2 were detected in the cultures of all 3 strains of *A. parasiticus*, and both AFL-A and AFL-B were detected in cultures inoculated with one of these strains. AF Q_1 and AF P_1 were not detected in any cultures inoculated with *A. flavus* or *A. parasiticus*.

In most of the cultures incubated for 15 d, AFs were detected most abundantly, after which they tended to decrease. However, AFLs continued to increase until the 20th day. The production patterns of AFLs were distinct from those of AFs. Analytical results of AFs and AFLs in cultures inoculated with each strain are shown in Table I.

The correlation between AF B_1 production and that of AF B_2 , AF M_1 and AFLs in 18 strains of *A. flavus* was studied on the 15th day of incubation. Production of AF B_2 was nearly 1/20 to 1/10 of AF B_1 , while that of AF M_1 was about 1/100 to 1/200 of AF B_1 . The correlation coefficient between AF B_1 and AF B_2 was fairly high at 0.957 and that between AF B_1 and AF M_1 was also reasonably noteworthy at 0.864. Furthermore, when the production of each toxin was studied over time, AF M_1 and AF M_2 had almost identical patterns to those of AF B_1 and AF B_2 . Yabe *et al.*^{18,19)} have reported that AF B_1 and AF G_1 are synthesized from dimethyl sterigmatocystin, and that AF B_2 and AF G_2 are synthesized from dihydrodimethyl sterigmatocystin in the course of biosynthesis, and that the same enzyme group most probably participates in both these reactions. The course of AF M_1 and AF M_2 biosynthesis, however, remains unclear.²⁰⁾ These results suggest that AF M_1

TABLE I. Production of Aflatoxins and Aflatoxicols by *Aspergillus flavus* and *A. parasiticus*

Fungus	Strain No.	Source	Incubation time(d)	Aflatoxins (ng/ml)						Aflatoxicols (ng/ml)	
				B ₁	B ₂	G ₁	G ₂	M ₁	M ₂	A	B
<i>Aspergillus flavus</i>	AF-144	Buckwheat flour	5	2030	92.7	ND	ND	17.0	Tr	ND	ND
			10	19000	1720	ND	ND	189	12.0	2.8	1.7
			15	23000	2170	ND	ND	226	18.3	4.7	3.6
			20	22800	2080	ND	ND	237	18.1	16.3	10.7
"	AF-145	Rye flour	5	2130	112	ND	ND	16.0	Tr	ND	ND
			10	34100	3200	ND	ND	400	30.9	2.4	1.4
			15	37700	4030	ND	ND	768	62.3	10.6	4.8
			20	19400	2280	ND	ND	643	51.0	9.0	5.5
"	AF-146	Buckwheat flour	5	3490	111	ND	ND	16.0	Tr	ND	ND
			10	28400	2200	ND	ND	280	26.2	2.5	1.4
			15	32200	3300	ND	ND	354	38.9	10.6	8.8
			20	29700	2850	ND	ND	291	22.0	19.1	12.5
"	AF-147	Maize	5	4010	124	ND	ND	18.5	Tr	ND	ND
			10	13700	997	ND	ND	111	Tr	3.8	2.2
			15	28500	2740	ND	ND	259	17.7	6.5	2.6
			20	22000	1860	ND	ND	192	13.0	9.9	6.4
"	AF-149	Job's tears	5	2.4	ND	ND	ND	ND	ND	ND	ND
			10	255	7.7	ND	ND	2.1	ND	ND	ND
			15	281	8.2	ND	ND	2.5	ND	ND	ND
			20	193	10.1	ND	ND	1.7	ND	ND	ND
"	AF-150	Job's tears	5	42300	1570	ND	ND	301	21.0	Tr	ND
			10	65100	3760	ND	ND	490	29.0	20.2	7.2
			15	92300	7200	ND	ND	705	64.0	91.8	46.1
			20	95200	6830	ND	ND	726	72.8	151.2	75.9
"	AF-151	Buckwheat flour	5	189	1.5	ND	ND	ND	ND	ND	ND
			10	651	25.0	ND	ND	6.3	ND	1.4	0.5
			15	2210	119	ND	ND	29.9	Tr	4.7	3.8
			20	1060	55.4	ND	ND	17.4	Tr	8.7	6.3
"	AF-152	Butter bean	5	1.2	ND	ND	ND	ND	ND	ND	ND
			10	10.8	2.4	ND	ND	ND	ND	ND	ND
			15	37.4	3.0	ND	ND	ND	ND	ND	ND
			20	41.8	3.5	ND	ND	ND	ND	ND	ND
"	AF-153	Job's tears	5	213	2.6	ND	ND	Tr	ND	ND	ND
			10	290	6.0	ND	ND	1.6	ND	ND	ND
			15	1310	24.1	ND	ND	9.7	ND	Tr	ND
			20	697	16.6	ND	ND	6.2	ND	Tr	Tr
"	AF-154	Peanut	5	34.5	2.2	ND	ND	ND	ND	ND	ND
			10	5760	341	ND	ND	53.7	4.7	5.0	2.4
			15	6510	380	ND	ND	62.9	4.5	11.2	9.8
			20	4920	326	ND	ND	40.7	3.5	15.8	13.2
"	AF-155	Kidney bean	5	129	5.0	ND	ND	Tr	ND	ND	ND
			10	5030	218	ND	ND	53.7	2.2	1.6	0.6
			15	5760	257	ND	ND	68.2	3.8	1.8	1.9
			20	5580	289	ND	ND	68.6	3.7	7.2	6.9

ND: Not detected

Tr: <1.0 ng/ml

TABLE I. Continued

Fungus	Strain No.	Source	Incubation time(d)	Aflatoxins(ng/ml)						Aflatoxicols(ng/ml)	
				B ₁	B ₂	G ₁	G ₂	M ₁	M ₂	A	B
<i>Aspergillus flavus</i>	AF-156	Paprika	5	46.9	1.9	ND	ND	Tr	ND	ND	ND
			10	4490	158	ND	ND	40.1	Tr	1.0	Tr
			15	6550	298	ND	ND	65.0	3.6	2.6	1.7
			20	4080	165	ND	ND	40.0	2.6	6.9	4.4
	AF-157	Peanut	5	2460	169	ND	ND	32.0	Tr	ND	ND
			10	21900	2180	ND	ND	268	40.0	1.2	Tr
			15	23100	2830	ND	ND	299	50.8	6.0	3.6
			20	27100	3310	ND	ND	370	63.3	17.5	12.5
	AF-158	Peanut	5	17.8	ND	ND	ND	ND	ND	ND	ND
			10	110	3.5	ND	ND	ND	ND	ND	ND
			15	210	18.6	ND	ND	ND	ND	ND	ND
			20	189	20.7	ND	ND	ND	ND	ND	ND
	AF-159	Nutmeg	5	3.6	ND	ND	ND	ND	ND	ND	ND
			10	1590	50.1	ND	ND	4.5	ND	Tr	ND
			15	4080	157	ND	ND	19.1	ND	Tr	Tr
			20	3730	150	ND	ND	20.3	ND	8.0	3.7
	AF-160	Nutmeg	5	40.6	Tr	ND	ND	ND	ND	ND	ND
			10	78.0	4.3	ND	ND	ND	ND	ND	ND
			15	87.0	8.9	ND	ND	ND	ND	ND	ND
			20	66.5	10.6	ND	ND	ND	ND	ND	ND
	C-35-a	Corn flour	5	10.9	ND	ND	ND	ND	ND	ND	ND
			10	642	53.1	ND	ND	3.5	ND	ND	ND
			15	43600	1920	ND	ND	221	9.4	60.4	49.0
			20	47100	2830	ND	ND	261	24.2	302	264
	C-39-a	Corn flour	5	373	17.5	ND	ND	2.3	ND	5.3	4.4
			10	5510	363	ND	ND	67.1	5.3	210	210
			15	7600	442	ND	ND	91.0	8.4	791	474
			20	7480	399	ND	ND	70.3	8.1	980	546
<i>Aspergillus parasiticus</i>	AF-105	Pistachio nuts	5	3850	175	17700	919	164	57.3	ND	ND
			10	4730	287	33600	2230	323	364	6.3	6.6
			15	7380	511	43600	3180	414	698	12.0	10.7
			20	2770	204	10700	872	153	68.7	8.4	6.0
	AF-120	Pistachio nuts	5	126	7.7	6.0	Tr	ND	ND	ND	ND
			10	872	36.4	112	9.2	9.7	9.0	ND	ND
			15	504	24.1	59.2	5.9	5.5	3.0	ND	ND
			20	133	9.4	7.7	Tr	ND	ND	ND	ND
	C-41-d	Maize	5	209	8.7	1470	67.9	8.7	7.4	ND	ND
			10	483	21.9	3830	210	23.2	134	ND	ND
			15	510	35.2	2520	217	49.7	176	ND	ND
			20	332	27.6	1570	138	13.8	261	ND	ND

ND: Not detected

Tr: <1.0 ng/ml

and AF M₂ were not biosynthesized by metabolism from AF B₁ and AF B₂, but by other channels from different precursors.

There have been several reports regarding production of AF B₁, AF B₂, AF G₁, AF G₂, AF M₁ and AF M₂ by *A. flavus* and *A. parasiticus*. However, as to the production of

AFL by these fungi, there is only our previous report¹⁰⁾ referring to the strain isolated from AFL-contaminated maize. Frequency of isolation of AFL-producing strains has not been determined. In this study using strains of different origins isolated from various foods, AFL was generally produced by AF-

Fig. 4. Mass Spectrum of Aflatoxicols in extracts from cultures inoculated with *Aspergillus flavus* (AF-150 strain)

(A) : aflatoxicol A, (B) : aflatoxicol B.

producing *A. flavus*. Three out of 18 strains produced comparatively abundant AFL (> 100 ng/ml), however, in cultures of the other 15 strains, very small quantities of AFL were detected. Moreover, AFL levels in culture tended to increase, although AFs tended to decrease on the 20th day of incubation, and AFLs tended to appear after AFs had accumulated in culture. Therefore, no correlation between AFL and AF B₁ production was observed. From this finding, it was presumed that AFLs in culture were converted from AF B₁. We found the same result in an AFL productivity test¹⁰⁾ using *A. flavus* isolated from AFL-contaminated maize. Confirmation of AFL-A and AFL-B produced by *A. flavus* was carried out by MS using extracts from cultures of AF-150, C-35-a and C-39-a strains.

AFL-A and AFL-B obtained from a mould culture had mass spectra which corresponded well with that of the reference standard (Fig. 4).

3. Metabolic Conversion of AF B₁ by Non-AF-Producing *A. flavus*

Conversion of AF B₁ to the hydroxide metabolites, AF M₁, AF P₁, AF Q₁, AFL-A and AFL-B, was studied in non-AF-producing *A. flavus* (Table II).

AF B₁ added to the cultures was metabolized or degraded by all strains. Fifty percent of the AF B₁ added remained on the 10th day and approximately 25% on the 20th day. When AF B₁ metabolites were analyzed, AFL-A and AFL-B were detected in all of the cultures inoculated with non-AF-producing strains. AFL levels were very low in the cultures of 6 strains, except in that of AF-141 (which had possessed AF B₁ productivity at initial isolation, but lost it upon subculture), in which approximately 250 ng/ml of AFLs was detected on the 15th day of incubation. This corresponded with about 12% of the AF B₁ added. However, on the 20th day of incubation, AFL levels were reduced in nearly all the strains (5 strains), which indicates that the produced AFL was incorporated by the fungi for further metabolism. These facts support the aforementioned hypothesis that AFLs manufactured by the AF-producing *A. flavus* were converted from AF B₁ by its own metabolic sequences. AF M₁, AF P₁ and AF Q₁, however, were not detected from any cultures of any strain.

From our metabolic studies of AF B₁ using non-AF-producing strains, only one strain out of 7 was capable of converting AF B₁ to AFL. Further, in AF-producing strains, 3 out of 18 produced AFL comparatively abundantly. This corresponded to approximately 17% of tested AF-producing strains.

TABLE II. Conversion of Aflatoxin B₁ to Aflatoxins by Non-Aflatoxin-Producing *Aspergillus flavus* (ng/ml)

Strain No.	Source	Incubation time (d)	Residual amount of aflatoxin B ₁	Detected amount of aflatoxins	
				A	B
AF-141	Biscuit	5	1600	33.5	37.7
		10	827	87.3	102.5
		15	707	136.0	115.7
		20	662	95.7	99.6
AF-142	Buckwheat flour	5	1530	2.8	1.9
		10	1080	4.2	2.6
		15	564	18.1	5.1
		20	357	10.8	2.4
AF-143	Bread	5	1220	1.3	Tr
		10	786	13.3	4.0
		15	525	7.7	2.6
		20	515	6.3	3.4
AF-148	Nutmeg	5	1540	1.2	Tr
		10	1230	18.1	6.0
		15	912	21.7	6.4
		20	510	11.7	4.4
C-40-e	Maize	5	1230	2.1	Tr
		10	963	1.8	1.0
		15	745	13.5	8.2
		20	469	5.2	1.5
C-40-f	Maize	5	1120	2.0	2.1
		10	850	5.2	5.8
		15	710	4.1	2.6
		20	625	9.9	3.4
C-40-j	Maize	5	1306	1.7	1.6
		10	855	Tr	Tr
		15	764	6.0	3.7
		20	456	7.3	3.3

Tr: 1.0 ng/ml

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