영남지방 농산물에 대한 위생학적 연구
(제 1보) Aflatoxin 생성균의 분리

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Hygienic Studies on Agricultural Products in Youngnam Districts
(Part I) Isolation of Aflatoxin Producing Strains

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ABSTRACT—To isolate the aflatoxin producing strains from agricultural products in Youngnam districts, rice(59), meju(30), corn(32), barley(58), soil(33), peanut(30), soybean(45), and unhulled barley(60) were collected from markets or homes. From 342 sample sources, 280 strains of *Aspergillus* spp. were isolated. As a result of screening by TLC, 29 strains expressed fluorescent spot and four strains have a same Rf value of standard aflatoxins, and the percentage of contamination from aflatoxin producing strains was 1.3%, and those strains were estimated as *Aspergillus flavus* group by the examine of characteristics and morphology.

Keywords □ **Aflatoxin B1, *Aspergillus flavus, Aspergillus parasiticus.***

Nowadays together with a fast advance of a natural economy in our country, a movement to improve food life was widespread, and as a result of this situations, a safety in agricultural products has taken an increasing interest to people in Korea. But any data to suitably evaluate agricultural commodities about its safety have not been systematically investigated untill nowadays. As a part of those work, hygienic studies on agriculcural products in Youngnam districts were attempted. Among many things Aflatoxin B1 was our first concern to study.

The aflatoxins were a family of potent mycotoxins produced by certain strains of the common molds, *Aspergillus flavus* and *Aspergillus parasiticus*[1]. Contamination of aflatoxin in food and feeds presented an important toxicological hazard[2,3].

The naturally occurring major aflatoxins were called aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2. Most toxigenic strains of *Aspergillus parasiticus* produced all four major aflatoxins while toxigenic strains of *Aspergillus flavus* produced only aflatoxin B1 aflatoxin B2[4,5]. Aflatoxin in groups exhibited strong blue fluorescence when viewed under ultraviolet light[6]. It formed colorless crystals and showed molecular weight about 312 to 344, and ultraviolet absorption showed 312-344 and 362-366 nm[7], respectively. Aflatoxins in the dry state were very stable to heat up to the melting point. However, in the presence of moisture and at elevated temperature there was

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Table 1. Location of sampling site and source of samples in Youngnam Districts

Source	Total	Location
Rice	59	*A(7), *B(8), *C(8), *D(7), *E(7), *F(7), *G(7), *H(8)
Meju	30	A(4), B(4), C(4), D(3), E(4), F(4), G(4), H(3)
Corn	32	A(4), B(4), C(4), D(3), E(5), F(4), G(4), H(4)
Barley	58	A(8), B(7), C(7), D(7), E(7), F(7), G(7), H(8)
Soil	33	A(4), B(4), C(4), D(4), E(4), F(4), G(4), H(5)
Peanut	30	A(5), B(3), C(4), D(3), E(5), F(3), G(2), H(5)
Soybean	45	A(6), B(5), C(5), D(5), E(6), F(6), G(6), H(6)
Unhulled barley	60	A(8), B(7), C(7), D(7), E(7), F(8), G(8), H(8)

*A: Sangju, *B: Porhang, *C: Chinju, *D: Kimhae *E: Milyang, *F: Ulsan, *G: Hamyang *H: Masan

a destruction of aflatoxin according to period of time. Outbreaks of aflatoxins in human and animals were closely related to consumption of food and feeds contaminated with aflatoxin producing strains[8,9]. At this point, many articles were published about aflatoxin contamination from all over the world[10-15]. Meanwhile, in our country only few study[16-18] on aflatoxin was reported in agricultural products from some area, but really not sufficient.

In this present paper, we isolated and screened aflatoxin producing strains from agricultural products such as rice, meju, corn, barley, peanut, soybean and soil in Youngnam districts.

MATERIALS AND METHODS

Sample source—A total of 342 samples were collected during march to july, 1989 from each area in Youngnam districts. Sample grains collected were 8 kinds as can be seen at Table 1: rice(59), corn(32), barley(58), peanut(30), soybean(45) meju(25), unhulled barley(6) and soil(33). In sampling, some cereal grains were mouldy or deteriorated because our first purpose was in isolation of aflatoxin-producing strains.

Media and reagent—For initial isolation from cereal grains, rose-bengal agar medium was used. Especially, rose-bengal, antibacterial agent, was added 35 mg/*l*. Therefore it can be used for pure isolation of *Aspergillus* spp. by preventing bacteria growth. The composition could be seen at Table 2.

Table 2. The composition of rose bengal medium

Glucose	10g
Peptone	5g
KH_2PO_4	1g
$MgSO_4 \cdot 7H_2O$	0.5g
Rose bengal	35 mg
Agar	15g
Tetracycline	35 mg
Distilled water	1 *l*

Table 3. The composition of modified PDA medium

Potatoes	200g
Bacto-dextrose	20g
Bacto-agar	15g
Yeast-extract	50g
Distilled water	1 *l*

The pH of the medium was adjusted to 5.2 with 25 percent ammonia and once the medium had been autoclaved, rose-bengal and tetracyline were added. To maintain *Aspergillus* spp. and isolate PDA adding 0.5% yeast-extract (pH 5.3) were used.

The composition was as follows (Table 3) and YES broth (yeast extract 2% and sucrose 18%) and SLS broth were employed for the growth and aflatoxin production. The composition of SLS medium was described in Table 4.

Aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2 were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A), and all inorganic

Table 4. The composition of modified SLS medium

Sucrose	85g
L-Asparagine	10g
$(NH_4)_2SO_4$	2g
KH_2PO_4	2g
$MgSO_4 \cdot 6H_2O$	1g
$CaCl_2 \cdot 2H_2O$	75 mg
$ZnSO_4$	10 mg
$Na_2B_4O_7$	2 mg
$FeSO_4 \cdot 6H_2O$	2 mg
$MnCl_2 \cdot 4H_2O$	5 mg
Ammonium molybdate	2 mg
Distilled water	1 *l*

*Initial pH of the medium was 4.5

chemicals and organic solvents used through the study were reagent grade or better.

Pure isolation—1g of samples transferred into 10 m*l* of distilled water in a test tube and then mixed by vortex mixer. They were cultivated on rose-bengal agar medium by streak plate method with glass rod and incubate the petri dish at 28°C for 3 days. Strains which were morphologically similar to *Aspergillus* spp. were maintained on potato dextrose agar (PDA) slants. Consequently, 280 strains from 342 samples were isolated in order to detect aflatoxin-producing strains.

Preparation of spore suspension—The mold was grown on PDA medium for 2 weeks at room temperature. Spores were harvested by adding 10 m*l* of 0.1% tween 80 to the PDA medium and gently bruching the conidiophores with a strerile inoculating loop. This procedure was repeated twice and the harvested spore suspension was pooled. The spore suspension was then filtered through four layers of sterile cheesecloth and diluted with sterile phosphate buffer to a concentration of approximately 10^7-10^8 conidia/m*l*. Spore number was determined by microscopic technique.

Inoculation and incubation—Tip culture method was used for the aflatoxin production of strains isolated in YES or SLS broth. A chip of the pippetman tip was stuffed with glasswool. The tip was

Spot of cultured media on TLC plate
↓
Place enough solvent in separating chamber to 1.4 cm
↓
Stopper
↓
Develop (chloroform/acetone = 9/1, v/v)
↓
Determination under UV light (365 nm)

Fig. 1. Analytical method of aflatoxin by TLC

placed in a glass tube (9 mm in diameter, 110 mm in length) and the top of the tip was covered with cap. After the tube was autoclaved and dried, the bottom of the tip was shielded with parafilm. Autoclaved medium (250 μl) was poured into resultant tip, and spores were; inoculated with a sterile inoculating loop directly for the first screening, but for the second screening, 5 μl of the spore suspension was inoculated. The culture was grown for 7 days at 28°C in a tip box. After incubation, parafilm was removed from the tip, and the whole set was centrifuged at 900 rpm for 20 sec. and the filtrate was directly spotted on a silica gel plate for TLC.

Fluorescence on TLC—Aflatoxin group were determined by directly spotting of the filterates on thin layer chromatogrphy (TLC) plate. In detail, each filtered broth analyzed by the following methods. Spot final filterates (5 μl) and standard AFB1 together on TLC plate and then developed by using chloroform: Acetone (9:1) about 15 min in TLC chamber and then observed under longwave UV-Light (365 nm) for fluorescent bend as a Rf values. Analytical procedure of aflatoxin by TLC is summarized in Fig. 1.

Characteristics and morphology of screened strains—Strains which had aflatoxin-producing ability were inoculate on PDA medium for identification. By slide glass culture method in microscope, color, morphology, texture of colony, conidial head, conidiophores, visicles, sterigmata, and conidia were observed and then followed the texonomic schemes of Raper *et al.*[19]

Table 5. The distribution of the strains of *Asp. flavus* cultivated on SLS and YES, and examination for aflatoxin-producing stranins by UV-light and TLC

Source of samples	No. of samples	No. of isolated strains	No. of fluorescent strains	No. of aflatoxin producing-strains
Rice	59	80	8	-
Corn	32	14	3	-
Barley	58	28	1	-
Soybean	45	10	3	-
Meju	25	62	2	-
Peanut	30	17	6	2
Soil	33	16	-	-
Unhulled barley	60	53	6	2
Total	342	280	29	4

RESULTS AND DISCUSSION

Isolation of aflatoxin-producing strains—A total of 342 samples collected during march to July, 1989 were surveyed for their contamination of aflatoxin-producing strains. Thin-layer chromatography has been the most widely used analytical method for separating and identifying mycotoxins from concentrated extracts. The technique of TLC involves applying a concentrated sample on a base live, separation by solvent migration, drying and characterization of the resultant spots. As a result of TLC, we separated four aflatoxin-producing strains when compared Rf value and color of fluorescent bends. As can be seen in Table 5, among the 280 strains isolated, 29 strains showed fluorescent bend and four strains were aflatoxin-producing strains. The percentage of *A. flavus* positive samples was 1.3% in screening by TLC. It would be noted that the cereal grains in Youngnam districts could be suspected to be associated with field outbreaks of mycotoxicoses.

In detail, strain numbers of P-38, P-49, B-75, B-79 had the some Rf value as those of standard aflatoxin group. B-79 produced AFG1 and AFG2, meanwhile, P-38, P-49, and B-75 produced

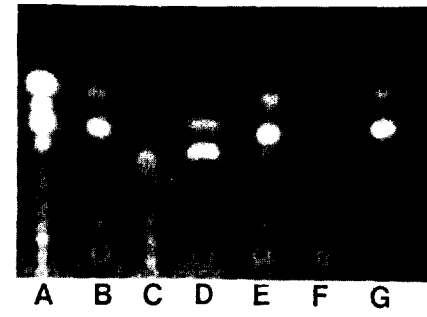

Fig. 2. TLC chromatograms of isolated strains.
A: Standard aflatoxin
B: P-38, C: C-27, D: B-79
E: P-49, F: R-270, G: B-75

Table 6. Morphology and physical property both B-79 and P-49 screened strains in Yongnam area

Observance	A*	B**
Colonies		
Rate of growth	Rapidly spreading	Rapidly spreading
Texture	Velvety	Velvety
Front	Greenish blue	Grayish blue
Reverse	Clear orange yellow	Yellow
Conidial head		
Color	Greenish gray blue	Greenish gray
Form	Compact columnar	Column
Size	250μm	60-320μm
Conidiophores		
Length	800-1,500μm	800-1,300μm
Width	13-15μm	7-16μm
Color	Colorless	Colorless
Visicles		
Shape	Globose	Globose
Size	15-40μm	17-38μm
Color	Gray	Gray
Sterigmata		
Color	Grayish yellow	Gray
Length	5-8μm	4-9μm
Width	3μm	2-7μm
Conidia		
Surface	Smoth	Smoth
Color	Yellow	Gray
Size	3-4μm	3-7μm
Shape	Semi-globose	Globose

*: B-79, **: P-38, P-49, and B-75

AFB1 as could be seen at Fig. 2.

Moreover, it was possible to detect easily the aflatoxins for a large number of strains by using the tip culture method. A method for the screening of strains had been reported in which the toxin was produced in liquid medium, followed by some purification procedures and final detection by TLC. However, the tip culture method described herein was much simpler than those culture and the amount of organic solvent required in this method was very small. Used tip containing mycelia and parafilms should be detoxificated and the tip culture method could also be applied to other molds. Matilde *et al.*[20] also screened aflatoxin-producing strains from mixture feed and cereal grain in Spain and suggested the glucose-yeast extract agar (GYA) medium was very suitable for screening aflatoxin-producing strains.

Morphology and characterization—Morphology of strains finally screened strain expressed in Table 6. When compared classification of Raper *et al.*[19] the strains of B-79, P-39, P-49, and B-75 have the same mode action of *Aspergillus flavus* group. We estimated those strains as *Aspergillus flavus* group. The present results indicated that aflatoxigenic strains could be occurred in several districts in Korea, and more study in this field were needed.

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국문요약

영남지방의 농산물로부터 aflatoxin 생성균을 분리하기 위해 쌀 59점, 메주 30점, 옥수수 32점, 보리 58점, 토양 33점, 땅콩 30점, 대두 45점 및 겉보리 60점을 1989년 3월에서 7월 사이에 수집하였다. 총 342 시료로부터 280종의 *Aspergillus* 속 균주를 분리하였고, 그 중에서 29 균주가 형광성을 나타내었으나 4균주가 표준 aflatoxin과 Rf 치가 같은 물질을 생산하여 전체 시료의 1.3%가 aflatoxin 생성균에 오염되어 있음을 알 수 있었고 균학적 성질을 관찰한 결과 4균주 모두 *Asp. flavus* group으로 추정되었다.

REFERENCES

1. Henrik Stenwig *et al.*: Aflatoxin producing strains of *Aspergillus flavus* isolated from-feeds in Norway, *Acta Agric. Scand.*, **38**, 207-313 (1988).
2. Wogan, G.N. and P.M. Newberne: Dose-response characteristics of aflatoxin B1 carcinogenesis in the rat, *Cancer Res.* **62**, 1281-1284 (1979).
3. Busby Jr., W.F. and G.F. Wogan: Mycotosins and mycotoxicoses. In H. Rieman and F.L. Bryan(ed), Food-borne infection and intoxification, 2nd ed. Academic press, New York, pp.519-610 (1979).
4. Austwick, P.K.C.: Aflatoxicosis in poultry. In mycotoxic fungi, mycotoxins, mycotoxicoses: An encyclopedic handbood. Vol.2. Ed. by T.D. Wyllie and L.G. Morehouse. Marcel Dekker Inc., New York, pp.279-301 (1978).
5. Bodine, A.B., and Metens, D.R.: Toxicology, metabolism, and physiological effects of aflatoxin in the bovine. In aflatoxin and *Aspergillus flavus* in corn. Ed. by U.L. Diener, R.L. Asquith, and J.W. Dickens. Craftmaster Printers Inc., pelika, Alabama, pp.46-50 (1983).
6. Yabe, K., Hiroshi, Nakamura, Yoshiji, Ando, Nobuyuki Terakado *et al,*: Isolation and characterization of *Aspergillus parasiticus,* mutants with impaired aflatoxin by a novel tip culture method, Applied and Envirio. Microbiology, pp.2096-2100 (1988).
7. Cole, R.J. and Cox, R.H. Verrucarol.: In handbook of toxic fungal metabolites. Academic Press Inc., New York, pp.157-159 (1981).

8. Bamburg, J.R.: Chemical and biochemical studies of the trichothecene mycotoxins. In mycotoxins and other fungal related food problems. Ed. by J.V. Rodricks. *Adv. Chem. Series* **149,** pp.144-162 (1977).
9. Campbell, A.D., O.J. Francis Jr., R.A. Beebe and L. Stolfff: Determination of aflatoxins in peanut butter, using two liquid chromatographic methods., *J. Assoc. Off. Anal. Chem.*, **67,** 312-316 (1984).
10. Wei Yun J. Ts al, Jimmy D. Lambert and Loyd B. Bullerman: Simplified method for microscale production and quantification of aflatoxin in broth, *Jounal of Food Protein,* 526-529 (1984).
11. Roscoe L. Warner and James J. Pestka: ELISA survey of retail grain based food products for zearalenone and aflatoxin B1, *J. of Food Protection,* **50,** 502-503 (1987).
12. Edds, G.T. and Brotell, R.A.: Biological effects of aflatoxin-poultry. In Aflatoxin and *Aspergillus flavus* in corn. Ed. by U.L. Diner, R.L. Asquith and J.W. Dickens, Craftmaster Printers Inc., Opelika, Alabama, pp.56-61 (1983).
13. Christensen, C.M.: Fungi in cereal grains and their products. In Mycotoxins in feedstuffs. Ed. by G.N. Wogan. The M.I.T. Press. Massachusetts Institute of Technology, Cambridge, MA, (1965).
14. Chang, H.L., J.W. Devries and W.E. Hobbs: Comparative study of two methods for extraction of aflatoxin from peanut meal and peanut butter, *J. Assoc. Off. Anal. Chem.*, **62,** 1281-1284 (1979).
15. Ferguson-Foods, J. and J.D. Worran: Rapid HPLC determination of aflatoxin M1 and M2 in fluid milk, *J. Assoc. Off. Anal. Chem.*, **67,** 1111-1114 (1984).
16. Chung, D.H., J.K. Kim J.K. Jang and S.C. Choi: Studies on the inhibitor of aflatoxin production by *Aspergillus parasiticus* R-716, *J. Food Hygiene,* **1**(1), 23-29 (1986).
17. Chung, D.H., Y.C. Chung and N.K. Sung: Influence of Temperature Cycling on the Production of Aflatoxin in Solid Media, *Kor. J. Env. Hlth. Soc.*, **12**(1), 39-45 (1986).
18. Chung, D.H. and C.J. Kim: Studies on the Inhibitors on aflatoxin production by *Aspergillus parasiticus* R-716-Isolation of Aflatoxigenic Fungi and Effects of Initial pH & Zn-, *Kor. J. Appl. Microbiol. Bioeng.*, **14**(1), 9-16 (1986).
19. Raper, K.B. and D.I. Fennel.: The genus Aspergillus, Williams and Wilkins, Baltimore, Reprint, Krieger Pub. R.E. Hunginton, N.Y., (1915).
20. Matilde Carballo and Juana Autonia de Miguel: Rapid Detection of Aflatoxin-producing Strains of *Aspergillus flavus* group Isolated from Mixed feed and Cereal grain in Spain, *J. Sci. Food Agric.*, **40,** 11-15 (1987).