J. Sci. Soc. Thailand, 3 (1977), 42-51

A NEW TOXIGENIC STRAIN OF *ASPERGILLUS ALLIACEOUS*

T. GLINSUKON, W. THAMAVIT* and T. SITTIRACHA

Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand

(Received 20 December 1976)

Summary

*A new toxigenic strain of **Aspergillus alliaceous** species, **Aspergillus alliaceous** var **macrosterigmatus****, isolated from unpolished rice collected from Bangkabuer, Bangkok, is identified. On Czapek's solution (3% and 20% sucrose), M40Y and malt extract agar, the strain is characterized by conidia heads with biseriate sterigmata bearing short curved divergent conidia chains of globose conidia. The primary sterigmata are large, up to 30 μ in length, each bearing 3 small secondary sterigmata. Colonies on Czapek's solution agar grow slowly 3.5-3.7 cm at 25°C in 10 days but they grow rapidly on malt extract agar, 6.2-6.5 cm and M40Y agar, 8.5-9.0 cm. Incubation at higher temperature (37°C) inhibits the growth rate, 1.0-1.2 cm in 10 days.*

*A toxigenicity test was conducted with the crude toxin produced by **A. alliaceous** var **macrosterigmatus****. The weanling rats died within 2-3 days after oral administration of 270 mg/kg BW of the crude toxin. The pathologic lesions were observed in liver of treated rats. The most usual changes seen in liver were focal and massive necrosis without observable fatty infiltration in mostly midlobular and periportal zones. Pathogenic effect was not observed in mice injected intravenously with spore suspension of **A. alliaceous** var **macrosterigmatus****.*

Introduction

Seed-borne fungi are grouped as field and storage fungi according to time of invasion of the seeds, cereal grains and market foods and foodstuffs. The predominant storage fungi are *Aspergillus* and *Penicillium*[1,2]. *Aspergillus ochraceous* strains are predominant among other storage fungi of *Aspergillus flavus* and *Aspergillus niger* strains[2]. *A. ochraceous* Wilhelm produces ochratoxin A, a toxic dihydroisocoumarin metabolite along with ochratoxins B and C[3]. Ochratoxin A has been reported to have a high acute toxicity for duckling, rats and dogs[4-7]. This mycotoxin is known to cause kidney and hepatic damage and alterations in the intestine and lymphoid tissues. In 1975, a species of *Aspergillus* which belongs to *A. ochraceous* group was isolated during a survey on the population of toxigenic fungi in market foods and foodstuffs in Bangkok[2]. It is different from the described strain by Raper and Fennell[8] which has no sclerotia or cleistothecia but it has big club-like primary sterigmata. Furthermore, it produces olive to dark brown pigments. Since this isolate differs sufficiently to warrant its description as a new

*Present address: Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok.
**This name refers to a new isolate which being identified in the present study.

strain of *A. ochraceous* group, the purpose of this paper is then to describe the identity of this fungus and its toxigenicity in the rat.

Materials and Methods

Fungal Identification

All cultures were grown on various substrates as follows: Czapek's solution (3% sucrose), high sugar (20% sucrose) Czapek's solution (Difco Laboratories, Detroit, Mich.), M40Y and malt extract agar[8]. The cultures were incubated at 25, 30 and 37°C. The identity description of this fungus is based primarily on cultured characteristics and details of morphology found on Czapek's solution agar (3% sucrose) at 25° C. Characteristic observations from cultures on other substrates at different temperatures are also added. Color references are based on color plates in Ridgway's Color Standards and Color Nomenclature[9].

Toxigenic Bioassay

Production of the crude toxin

A crude toxin was prepared according to a modified method of Glinsukon and co-workers[10]. A portion of 5 ml of the spore suspension (2×10^8 conidia per ml) of *A. alliaceous* var *macrosterigmatus*** was inoculated into sterile glutinous rice (250 g) in water (250 ml) contained in a Fernback flask. The flasks were incubated at room temperature (25 ± 1° C) for 3 weeks. The moldy rice was then extracted three times with chloroform (750 ml) in Waring blender (1 gallon). An initial filtrate was collected through a Büchner funnel with Celite using a negative pressure. Additional filtration through anhydrous sodium sulfate removed the water and clarified the extract. The filtrate was reduced to the minimum volume in a vacuum rotary evaporator after which the oily residue was obtained. The oily residue was added slowly to swirling n-hexane [residue/n-hexane, 1/10 (V/V)] to precipitate the crude toxin. The complete precipitation was achieved by storing the n-hexane mixture at 4° C overnight. The crude toxin was recovered by filtration and dried *in vacuo*.

Toxicity test

Acute toxicity tests were conducted with the crude toxin administered to weanling male rats (40-50 g), Fischer derived strain, (Animal Production Center, Faculty of Science, Mahidol University). In all cases, three to five animals were used in each control and test groups. The crude toxin was dissolved in dimethylsulfoxide (DMSO, Burdick and Jackson Laboratories, Muskegon, Mich.) or Mazola corn oil (Best Foods, CPC International Inc., Englewood Cliffs, N.J.). The suspensions were administered intraperitoneally or orally to rats. Survivors and control animals were sacrificed 7 days after dosing, and autopsy was performed on these animals as well as on those that had died during the course of the experiment. At autopsy, gross examination was performed and samples of visceral organs were fixed in 10% neutral buffered formalin and processed by standard method for histopathologic examination.

Pathogenic Bioassay

Pathogenicity tests were performed by using the spore suspensions injected to adult male Swiss mice (25-30 g), (Animal Production Center, Faculty of Science, Mahidol University). Four mice were used in each control and test groups. The spore suspension (0.2 ml) at various concentrations of 2×10^4, 2×10^5 or 2×10^6 conidia per ml of 0.9% NaCl solution was injected intravenously to mice. Survivors and control animals were sacrificed at various time intervals. The autopsy was performed on these animals and visceral organs were fixed in 10% neutral buffered formalin, and processed by standard method for histopathologic examination.

Results

Fungal Description

Colonies on Czapek's solution agar (3% sucrose) growing slowly 3.5-3.7 cm in 10 days at 25°C and reaching a diameter of 4.2-4.5 cm in 2 weeks, consisting of a white to creamy yellow mycelium which submerged extensively (2.0-3.0 mm) beyond the edge of the surface growth, showing a circular wrinkle at the center with the radial furrows and sometimes showing more or less zonate (Fig. 1 a), producing more abundant conidia heads at the center which give the colony color of sea foam yellow (R., Plate XLI), ecru-olive (R., Plate XXX), raw sienna (R., Plate III) to buck-thorn brown (R., Plate XV) at the center with olive to drak brown circular rings (Fig. 1 c); *exudate* fairly abundant in clear droplets; *odor* lacking. *Conidia heads* variable in size ranging from 100-250 μ, at first globose with radiate conidia chains and later splitting into mostly straight compact divergent columns, in some others showing short curved columns (Fig. 2 b); *conidiophore* also variable in length up to 1.0-2.0 mm by 6.0-7.5 μ with yellow shade heavy wall (2.0 μ thick) showing smooth or fine echinulate to coarse of red granules; *vesicle* globose with thick wall (2.0-3.0 μ) and covered with fertile over its entire surface (Fig. 2 c); *sterigmata* fairly crowded and composed of two series, primaries commonly 15.0-28.0 μ by 3.0-5.0 μ but occasionally reaching 30 μ in length (Fig. 2 d) each bearing 2-3 secondary sterigmata, secondaries mostly 5.0-7.0 μ by 2.0-2.5 μ; *conidia* globose to subglobose (mostly 2.0-3.0 μ) with smooth to fine echinulate surface. Sclerotia were not observed.

Colonies on Czapek's solution agar (20% sucrose) growing rapidly 5.6-6.0 cm in 10 days and reaching 7.6-8.0 cm in 2 weeks, showing loose concentric zones with the compack conidia heads at the central areas, producing more abundant and crowded conidia heads than those found on Czapek's solution agar (3% sucrose) at the same incubated temperature, 25°C (Fig. 1 b). Other gross and microscopic characteristics were very similar to those described on Czapek's solution agar, except the reverse shows wide circular rings (Fig. 1 d).

Colonies on malt extract agar growing rapidly 6.2-6.5 cm in 10 days, usually uniform and velvety but sometimes showing loose concentric zones with less sporing and conidia heads more crowded at the margin.

Colonies on M40Y agar spreading broadly, attaining a diameter of 8.0-9.0 cm in 8-10 days, usually plane and velvety at the central areas with somewhat wide concentric

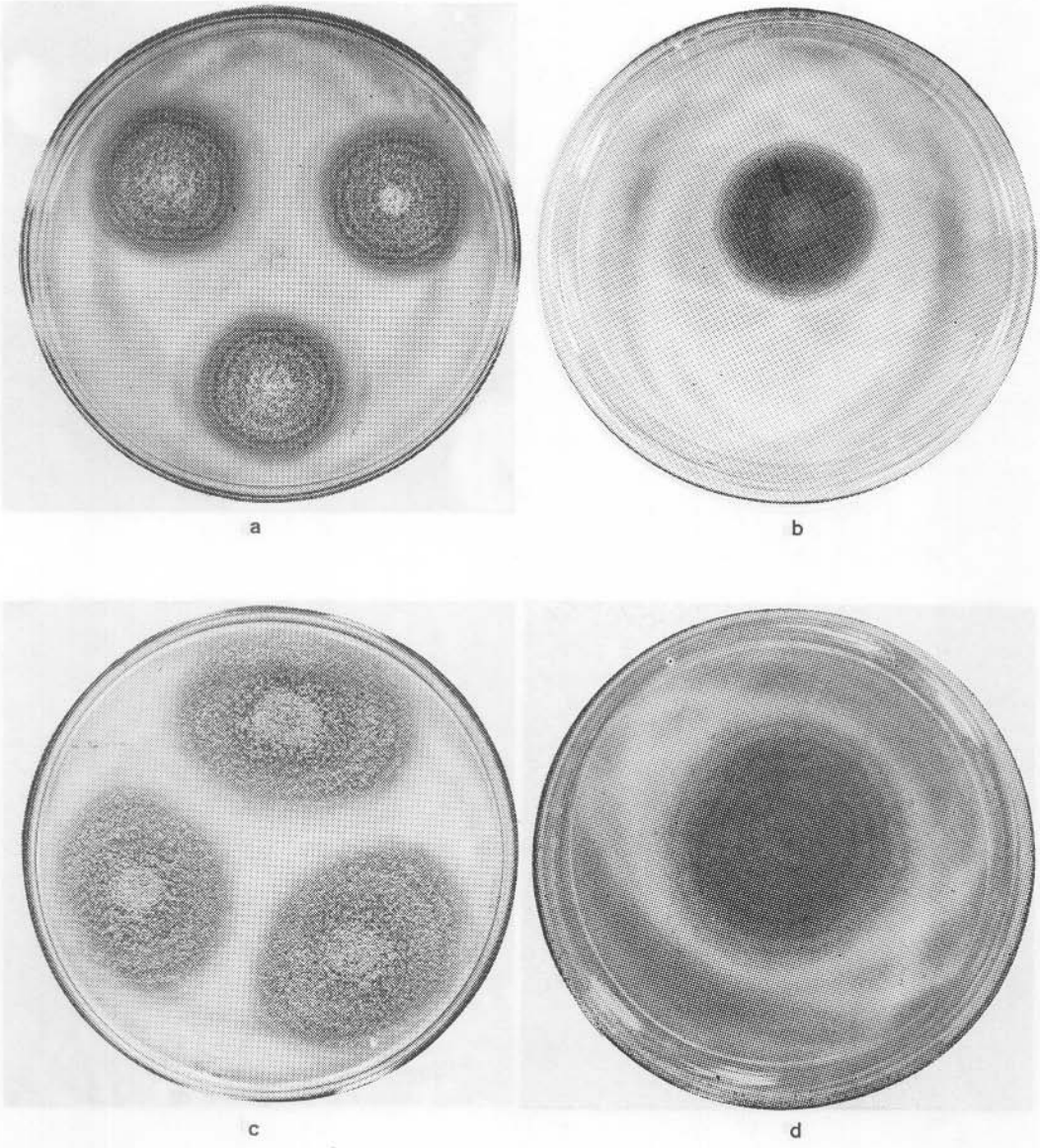

Fig. 1. Colonies of *Aspergillus alliaceous* var *macrosterigmatus*** on various substrates after the incubation for 10 days. On Czapek's solution agar (3% sucrose) at 25 °C; a, top view and b, reverse. On Czapek's solution agar (20% sucrose) at 25 °C; c, top view and d, reverse.

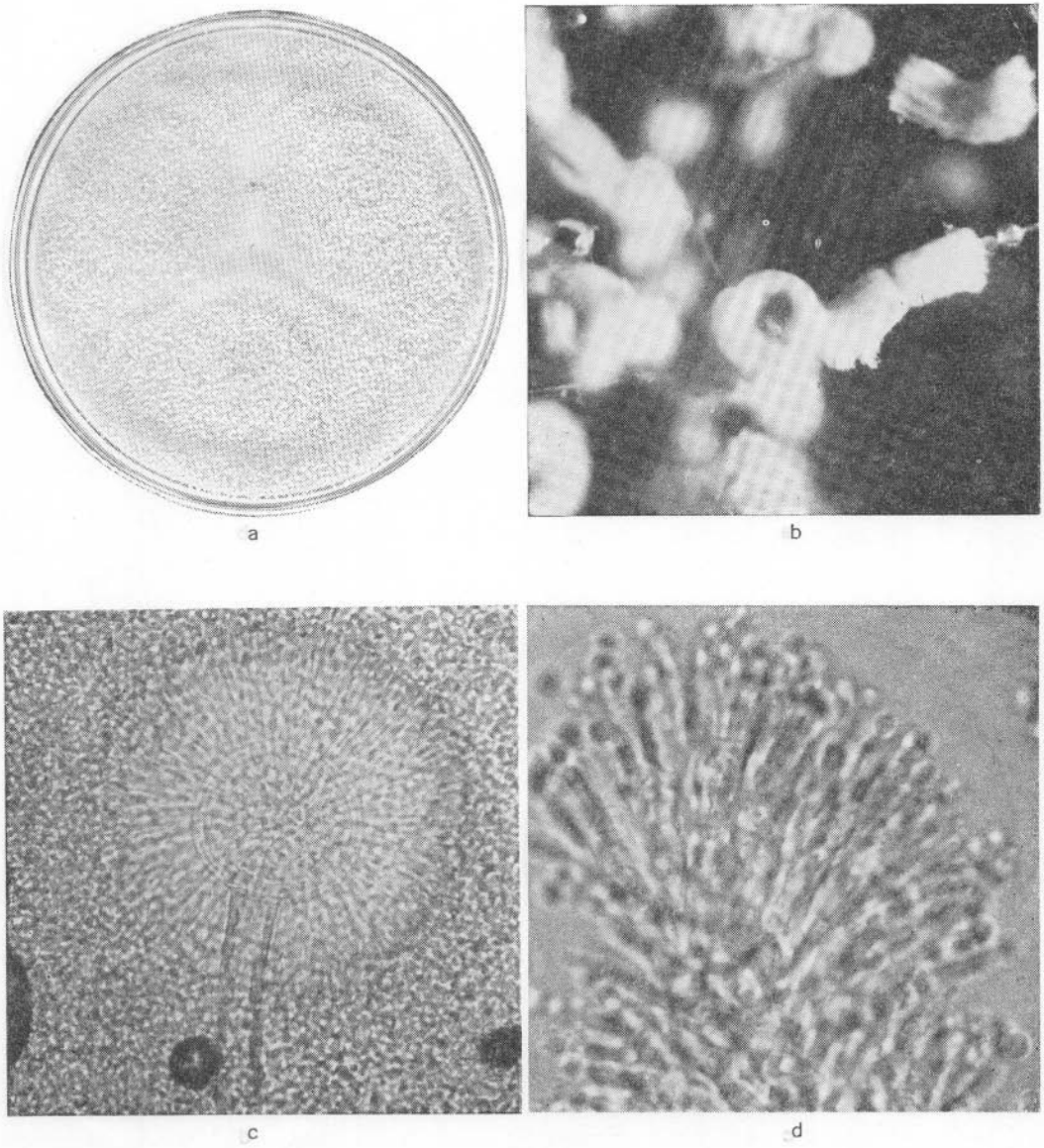

Fig. 2. Ten-day-old colonies of *Aspergillus alliaceous* var *macrosterigmatus*** on M40Y agar at 25 °C, a. Conidia heads on Czapek's solution agar at 25 °C, b. (40X). Details of the conidia structure, c. (400X). Details of the primary and secondary sterigmata, d. (1000X).

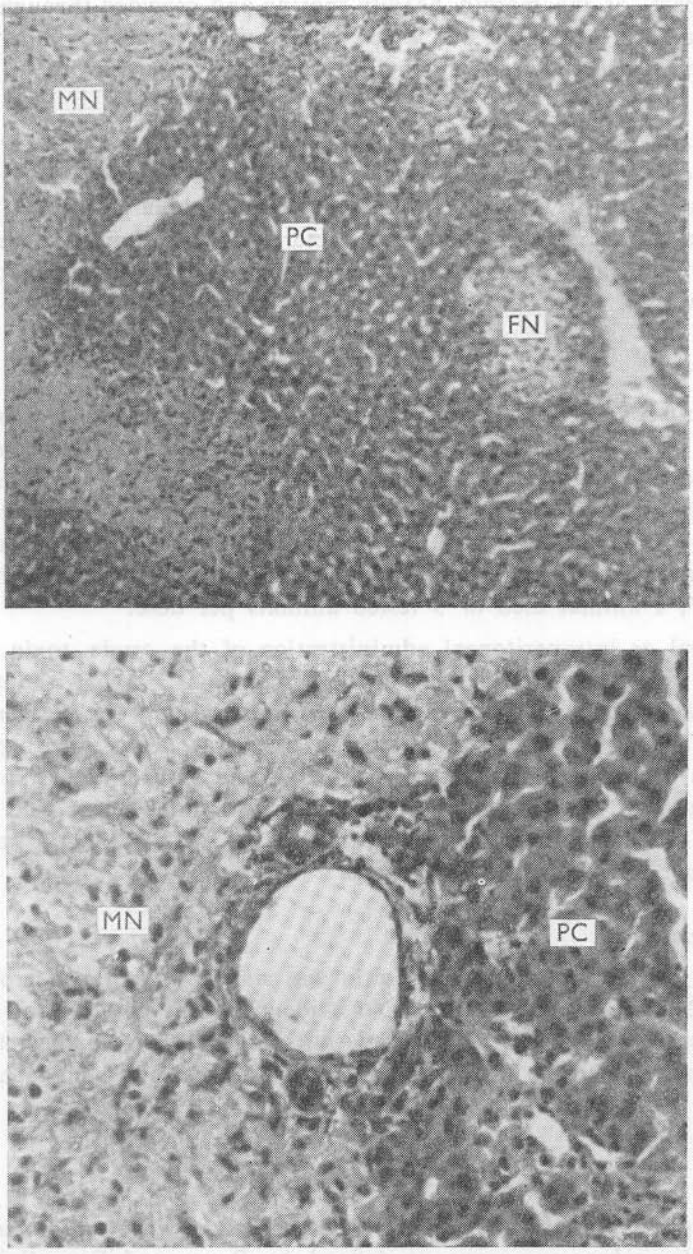

Fig. 3. Rat liver, rat treated with 270 mg/kg BW of the crude toxin and died 2 days after toxin administration (P.O.). a, Massive necrosis (MN), normal parenchymal cells (PC) and focal necrosis (FN) are shown (100X). b, Pycnotic nuclei and cell debris are observed in massive necrosis compared to normal parenchymal cells (PC) (250X).

zones (1 cm apart) at the margin, heavily sporing and crowded throughout. *Conidia heads* mostly radiate to globose and a few showing the conidia chains splitting into divergent columns; *conidiophores* much longer on this substrate up to 5.0 mm (Fig. 2 a).

At 37° C on Czapek's solution agar (3% sucrose) the conidial structures showing less crowded, growing very slowly 1.0-1.2 cm in 10 days, plane and thining towards the margin with vegetative mycelium largely submerged (1.0-1.1 cm) beyond the edge of the surface growth, other characteristics are similar to those described on Czapek's solution agar at 25° C, but the color of colonies somewhat lighter than sea foam yellow (R., Plate XXXI).

Fungal Toxigenicity

The results of the acute toxicity tests from a single oral or intraperitoneal administration of the crude toxin are summarized in Table I. In weanling rats, the crude toxin had a lethal dose of higher than 270 mg/kg BW administered orally and higher than 135 mg/kg BW administered intraperitoneally. According to these data, however, within a 7-day period after oral administration of 180 and 90 mg/kg BW, 1 and 1 animals died respectively and after intraperitoneal administration of 135 mg/kg BW, 1 animal died of 3 tested animals per dose.

After oral or intraperitoneal administration of the crude toxin, rats became sick, lost their appetite and weights, and died within 2-3 days without neurological symptoms. Histopathologic changes were seen qualitatively in both routes of administration. The most usual change seen in liver was necrosis with pynotic nuclei and cell debris originating in the periportal area and spreading to the midzone of the lobule (Fig. 3 a, b). Moreover, focal necrosis without observable fatty infiltration was also observed throughout the tissue (Fig, 2 a). Significant pathologic changes were not observed in other organs.

Fungal Pathogenicity

In adult mice, the body weight gain was similar in both control and treated groups after 120 days of inoculation. No evidence of intact spores of fungal germination was observed in the visceral organs within 14 days. Further, there were no significant changes observed in visceral organs with gross and histopathologic examinations.

Discussion

In the present study, *Aspergillus alliaceous* var *macrosterigmatus*** belongs to the *Aspergillus ochraceous* group as defined by Raper and Fennell in "*The genus Aspergillus*"[8]. *A. alliaceous* var *macrosterigmatus*** does not fit satisfactorily into any of the strains of *A. ochraceous* group. The strains most closely allied are *A. ochraceous* Wilhelm and *A. alliaceous*. The growth rate, pattern of growth and the characteristic of the conidia heads resembled those colonies of *A. ochraceous* Wilhelm with the exception of the whole colony color on Czapek's solution agar.

TABLE I: ACUTE TOXICITY OF THE CRUDE TOXIN FROM *A. ALLIACEOUS* var *MACROSTERIGMATUS*** IN WEANLING RATS.

Treatment[a]	Dose (mg/kg BW)	Route of administration	Dead/tested[b]		Survival time[c] (d)
			Crude toxin I	Crude toxin II	
Crude toxin	270	P.O.	2/3	3/5	2–3
Crude toxin	180	P.O.	1/3	2/5	2
Crude toxin	90	P.O.	1/3	1/5	2
Corn oil	1.0 ml	P.O.	0/3	0/5	Sacrificed
Crude toxin	135	I.P.	1/3	–	2
Crude toxin	90	I.P.	0/3	–	Sacrificed
Crude toxin	45	I.P.	0/3	–	Sacrificed
DMSO	0.1 ml	I.P.	0/3	–	Sacrificed

[a]Oral administration (P.O.), the crude toxin was dissolved in corn oil (1 ml/rat) and intubated via stomach tube. Intraperitoneal administration (I.P.), the crude toxin was dissolved in dimethylsulfoxide (DMSO, 0.1 ml/rat).

[b]Crude toxin I and II were prepared from two batches of moldy rice infested with *A. alliaceous* var *macrosterigmatus***.

[c]Survivors were sacrificed 7 days after dosing.

However, the growth rate on malt extract agar is similar to the growth rate of *A. alliaceous*. For the microscopic appearances, *A. alliaceous* var *macrosterigmatus*** shows thick walled vesicle, big club-like primary sterigmata bearing 3 small secondary sterigmata and the small size of conidia. These characteristics are strikingly similar to *A. alliaceous*, but the primary sterigmata are almost twice longer. Interestingly, sclerotia are not observed within 3 months of age on various substrates. This is a unique feature of *A. alliaceous* var *macrosterigmatus*** among the strains of *A. ochraceous* group, but with the exception of the only strain of *A. petrakii* Vörös which the production of sclerotia are not observed. It is not possible to classify this fungus as *A. petrakii* Vörös which has short primary sterigmata or *A. ochraceous* Wilhelm which has purple sclerotia. Therefore, *A. alliaceous* var *macrosterigmatus*** which is very similar to *A. alliaceous* should, be a new strain of *A. ochraceous* group. The generic name of the variety *macrosterigmatus* is selected to denote the big club like primary sterigmata produced by this fungus.

According to the acute toxicity data, it was indicated that the lethality of the crude toxin was at most increased one time when administered intraperitoneally. The small reduction in toxicity of the crude toxin by the oral route of administration is probably at least partially attributable to the chemical destruction under the acidic conditions presented in the stomach and/or toxin metabolism by gastrointestinal mucosal cells. This is considered to be very important in the role of the compound as a food-borne mycotoxin. Animals that died 2-3 days after the crude toxin administration had no convulsion or other neurological symptoms, but

mostly necrosis in liver. It is suggested that the crude toxin contains active compound which is considered to be a hepatotoxin. It was reported that *A. alliaceous* produced kojic acid[11, 12]. After kojic acid administration, the mouse became prostrate, stretching its legs and tail, and breathing slowly and laboriously. Convulsions were also produced before death[13]. It is, therefore, unlikely that the crude toxin from *A. alliaceous* var *macrosterigmatus*** will contain kojic acid. Ochratoxins were isolated from *A. ochraceous* Wilhelm[3]. *A. alliaceous* var *macrosterigmatus***, a strain of *A. ochraceous* group, may produce ochratoxins. However, ochratoxin A produced the fatty infiltration in liver of treated rat[4] whereas liver necrosis without observable fatty infiltration was seen in this study. Furthermore, no aflatoxins were detected in the crude toxin by thin layer chromatographic technic[14]. It appears, therefore, that *A. alliaceous* var *macrosterigmatus*** may produce a novel mycotoxin; however, the production of other known mycotoxins cannot yet be ruled out as there may be a species difference in mycotoxin production. According to the pathogenic tests, *A. alliaceous* var *macrosterigmatus*** did not show any pathogenic effect to mice.

Acknowledgements

The authors would like to thanks to Miss M.L. Busara Nuntavan and Mr. Sureesuk Karnchanasatid for their technical assistance, and Miss Wilai Limpasuk for her secretarial assistance. This work was supported in part by the grants from ASEAN and National Research Council, Thailand.

References

1. Christensen, C.M. (1965) in *Mycotoxins in Foodstuffs.* (Wogan, G.N., ed.) p. 9. M.I.T. Press, Cambridge, Massachusetts.
2. Glinsukon, T., Thamavit, W. and Ruchirawat, M. (1976) *J. Sci. Soc. Thailand* **2**, 176.
3. Van der Merwe, K.J., Steyn, P.S., Fourie, L., Scott, De B. and Theron, J.J. (1965). *Nature* **205**, 1112.
4. Theron, J.J., van der Merwe, K.J., Liebenberg, N., Joubert, H.J.B. and Nel, W. (1966) *J. Pathol. Bacteriol.* **9**, 521.
5. Purchase, I.F.H. and Theron, J.J. (1968) *Food Cosmet. Toxicol.* **6**, 479.
6. Purchase, I.F.H. and van der Watt, J.J. (1971) *Food Cosmet. Toxicol.* **9**, 681.
7. Szczech, G.M., Carlton, W.W. and Tuite, J. (1973 b) *Vet. Pathol.* **10**, 347.
8. Raper, K.B. and Fennell, D.I. (1965) in *The genus Aspergillus*, Williams and Wilkins Co., Baltimore, Maryland.
9. Ridgway, R. (1912) in *Color Standards and Color Nomenclature*, Published by author, Washington, D.C.
10. Glinsukon, T., Yuan, S.S., Wightman, R., Kitaura, Y., Büchi, G., Shank, R.C., Wogan, G.N. and Christensen, C.M. (1973). *Plant Foods for Man* **1**, 113.
11. Gill-Carey, D.E. (1949 a) *Brit. J. Exp. Pathol.* **30**, 114.
12. Gill-Carey, D.E. (1949 b) *Brit. J. Exp. Pathol.* **30**, 119.
13. Spector, W.S. (1957) in *Handbook of Toxicology. II Antibiotics*, Saunders, Philadelphia.
14. Eppley, R.M. (1968) *J. Ass. Off. Anal. Chem.* **51**, 74.

บทคัดย่อ

เชื้อราที่สร้างสารพิษตัวใหม่ในพวก แอสเปอร์จิลลัส อัลเลียเซียส ซีอแอสเปอร์จิลลัส อัลเลียเซียส แมคโครสเตอร์ริกมาตัส ขึ้นอยู่บนข้าวกล้อง จากตลาดบางกระบือ กรุงเทพมหานคร ได้ถูกนำมาจำแนกชนิดใหม่ เชื้อราตัวนี้เมื่อนำมาเลี้ยงบนวุ้นซาเบ็ค (3% และ 20% น้ำตาลซูโครส) เอ็ม 40 วาย และส่วนสะกัดจากข้าวม้อลท์จะมีลักษณะเด่นหลายประการ กล่าวคือมีสเตอร์ริกมาต้าเป็นสองตอนรองรับสปอร์ลักษณะกลมต่อกันเป็นกลุ่มของเส้นสปอร์ ซึ่งแยกออกจากกันเป็นลักษณะสั้นและโค้งทรงกระบอก ไพรมารี่ สเตอร์ริกมาต้าใหญ่ถึง 30 ไมครอนรองรับเซกัน สเตอร์ริกมาต้าอยู่ 3 อันด้วยกัน โคโลนีบนวุ้นซาเบ็คเจริญเติบโตช้า 3.5–3.7 ซม. ภายหลังที่เลี้ยงในห้องซึ่งมีอุณหภูมิ 25°ซ เป็นเวลา 10 วัน แต่จะเจริญเติบโตเร็วขึ้นบนวุ้นส่วนสะกัดจากข้าวมอลท์ 6.2- 6.5 ซม. และบนวุ้นเอ็ม 40 วาย 8.5–9.0 ซม. การเลี้ยงเชื้อที่อุณหภูมิสูง (37°ซ) จะสะกัดกั้นการเจริญเติบโตเหลือเพียง 1.0–1.2 ซม. ภายหลังที่เลี้ยงอยู่เป็นเวลา 10 วัน

การทดสอบการเป็นพิษได้กระทำโดยการนำสารพิษซึ่งสร้างจากเชื้อราแอสเปอร์จิลลัส อัลเลียเซียส แมคโครสเตอริกมาตัส ให้หนูขาวซึ่งอดนมใหม่ๆ กินด้วยหลอดกระเพาะในปริมาณ 270 มก/กก ของน้ำหนักตัว ภายหลังจากนั้น 2–3 วันหนูขาวจะตาย พบว่ามีการเปลี่ยนแปลงทางพยาธิสภาพในตับของหนูเหล่านี้ พยาธิสภาพที่เห็นชัดได้แก่การตายของเซลล์ของตับ ซึ่งพบเป็นกลุ่มเฉพาะที่และกลุ่มกว้างบริเวณระหว่างเส้นเลือดดำเซนทรัลและพื้นที่เพอริปอร์ตัลกับรอบๆ พื้นที่เพอริปอร์ตัลอีกด้วย แต่ไม่สามารถสังเกตเห็นการรวมตัวเป็นกลุ่มของไขมันภายในเซลล์เหล่านี้ นอกจากนี้ยังไม่พบพยาธิสภาพ อันเนื่องมาจากการฉีดสปอร์ของเชื้อราตัวนี้เข้าสู่เส้นเลือดของหนูขาวแต่อย่างใด