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Determination of Territrem B in Rice Media with Different Strains of *Aspergillus terreus* by Chromatography

SHIN-YUE FANG[1], QUE-KING WEI[1] AND FU-CHUO PENG[2]*

[1.] *Department of Food Nutrition , Chung-Hwa College of Medical Technology, Tainan Hsien, Taiwan, R.O.C.*

[2.] *Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan, R.O.C.*

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ABSTRACT

Territrem B is a tremogenic mycotoxin produced by *Aspergillus terreus*. Animal studies have demonstrated that territrem B caused whole body tremor, salivation, convulsion and death. Territrem B is also a potent irreversible inhibitor of acetylcholinesterase (AchE, E. C. 3.1.1.7). The aim of the present study is to assess the ability of strains of *Aspergillus terreus*s to produce territrem B in the rice media, which were obtained from Food Industry Research and Development Institute in Taiwan. In the 17 strains of *A. terreus*, only No. 32111 and 32664 strains produced territrem B-like blue fluorescence compound by TLC and HPLC analyses. Later on, this compound was identified as territrem B by mass spectroscopy. The amount of territrem B was determined by reverse phase HPLC on a C18 column with UV detection at 335 nm. The linear regression coefficient of the standard curve (Y=1428.5X+306.0) for territrem B at concentration ranging from 0 to 250 ng/L is 0.9998. In rice media, the recovery rates for territrem B at 50, 100 and 200 ng/L ranged from 91.5% to 95.2% with a coefficient of variation less than 2.1%. The relative standard of territrem B ranged between 1.6-2.8% (intra-day) and 0.6-2.3% (inter-day). In rice culture of *A. terreus* 32111 and 32664 strains, the contents of territrem B were 0.20 and 0.31 mg/100 g rice, respectively. From the above results, it can be concluded that this HPLC method for determination of territrem B in rice media is suitable and only domestic strains can produce territrem B.

Key words: Territrem, *Aspergillus terreus*, TLC, HPLC

INTRODUCTION

Many natural cereals, seeds, nuts and dried fruit are easily contaminated by fungus and are able to produce mycotoxin. The key types of mycotoxin are *Aspergillus*, *Penicillium* and *Fusarium*[(1)]. Among them, *Aspergillus* and *Penicillium* are the most commonly found in the contaminated foods after harvest and during storage. So far the known toxins include aflatoxin, ochratoxin, patulin, sterigmatocystin, and citrinin, etc. There can be as many as 300 types of mycotoxin produced from cultivation in the laboratories. However, if found in significant amount in food, only 20 types of these mycotoxin could raise safety concerns[(2)].

Rice is the staple grain in Taiwan. Locating in a subtropical climate region with high temperature and humidity, the environment is perfect for fungus to grow. There are constant incidences of mycotoxin poisoning[(3)] resulted from storaged grains being contaminated by microbes. Tung et al. in 1967 investigated the shall-on rice in storage that were contaminated by fungus and found that in the 206 strains, *Aspergillus* took up the majority number. Among the 206 strains, 11 were *A. terreus*[(4)]. A blue fluorescent material[(5)] with chemical structure similar to aflatoxin was found in No.23-1 *A terreus* strain. The material was later identified as territrem A (TRA), territrem B (TRB) and territrem C (TRC). Their only structural difference lies in their aromatic moeities. Among the three, territrem B had the highest content. The chemical formula is:

4a*R*,6a*R*,12a*S*,12b*S*)-4a,6,6a,12,12a,12b-hexahydro-4a,12a-dihydroxy-4,4,6a,12b-tetramethyl-9-(3,4,5-trimethoxy-phenyl)-4*H*,11*H*-naphtho(2,1-*b*)pyrano(3,4-*e*)pyran-1,11(5*H*)-dione,

and the molecular structure is $C_{29}H_{34}O_9$[(6)]. This toxin does not contain nitrogen and is a type of neurotoxin that induces symptoms, such as tremor, salivation, convulsion, liver or kidney congestion, and even death. Its key function is the territrem inhibitive effect on acetylcholinesterase[(7~10)] and its LD_{50} is 9 mg/kg body weight[(9)]. In order to understand the ability of different *A. terreus* in generating territrem B, we purchased from Hsin Chu Institute of Food Industry Development 17 types of *A terrus*, which were collected from different regions and countries for study. We would like to find out the connections between the territrem B productivity and the regions and countries from where the strains were collected.

Currently, there are many methods applied to analyze mycotoxin, such as Liquid Chromatography (LC), High Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC), Supercritical Fluid Chromatography (SFC), etc. For example, HPLC, TLC and Mass Spectroscopy (MS) are used in analyzing the ability of Taiwan *Fusarium* in generating fumonisins from corns[(11)]. HPLC is also used in analyzing phytotoxin[(12)] from *Leptosphaeria maculans* that damages brassica oilseed. Among these methods, HPLC is commonly used in analyzing aflatoxin[(13)]. Since territrem shares the similar structure

* Author for correspondence. Tel:886-2-23123456 ext. 8608 Fax:886-2-23915291; E-mail:fcpeng@ha.mc.ntu.edu.tw

as aflatoxin, HPLC analysis is incorporated with TLC and MS for territrem B identification and quantitative analysis.

MATERIALS AND METHODS

I. *Strains*

The strains used in the study were *Aspergillus terreus* CCRC30135, 31128, 32068, 32111, 32652, 32653, 32664, 32670, 32677, 32678, 21570, 32681, 32688, 32689, 32800 and 32877, etc. All 17 types were purchased at Hsin Chu Institute of Food Industry Development Preservation Center.

II. *Standard and Tester*

Chloroform, methanol, benzene, ethylacetate, acetonitrile, and formic acid were purchased from RdH Laborchemikalien GmbH & Co. KG, Germany. Territrem B standard was equationed[9] with purity over 99%.

III. *Method*

(I) *Preparing Inoculation Spores*

Based on the method developed by Ling *et al*[4], cultivated *A. terreus* strains were cultivated in the potato dextrose agar (PDA). After 7 to 10 days of cultivation, scrape and place the spores into sterilized water and added 0.01% TritonX-100 sterilized water. Calculate the spores by Petroff-Hauser[15] method for use during inoculation.

(II) *Extracting Territrem B*

Extract territrem B by the methods described by Ling *et al*[16]. Purchase rice from supermarkets and soak in water in 50 g flasks. Filter out the water through gauze the next day, sterilize in the sterilizing pot and then cool on the Laminar flow. Take around 10^8/ml of inoculation fluid with spores, add to the rice media, cultivated for 14 days under 28˚C and extract with 30 ml of chloroform for three times. Decompress the extracted fluid to dry with a decompressing condenser (N-N SERIES/WATERBATH SB-450/ASPIRATOR A-3S/TIT BL-20, TUNGTEC INSTRUMENTS CO., Taiwan). The condensed material was then solved with 5 ml of chloroform to be used as extract fluid for the following quantitative and qualitative analysis.

(III) *Qualitative and quantitative analysis on territrem B*

(1) Territrem B Qualitative Analysis

Pre-vitalizing the TLC board (Macherey-Nagel ART.-Nr818133, 0.20mm) for 2 hours in 80˚C based on Lin *et al*[17] method. Perform one-dimensional thin layer chromatography on the 0.1 ml chloroform extract and the territrem B standard, using benzene: ethylacetate at 1:1 ratio as the developing solvent system. After development, observe with UV light (ENF-280C, Spectronics CO. USA) and scrape the location with value equal to the Rf value of TRB. Extract with 3 ml of chloroform three times, let dry and then solve with 1 ml of methanol to perform HPLC analysis. Two-dimensional thin layer chromatography was performed first by one-dimensional developing system by using 10 ml of chloroform extract under benzene : ethylacetate at 1:1 ratio. Then use toluene: ethylacetate : formic acid at 5: 4: 1 ratio for a two-dimensional developing system. Use UV light to observe the extracted metabolism and calculate the Rf values.

(2) Testing territrem B

Put the purified territrem B, that was cultivated from No.32111 and 32664 in the rice media after purification (purified level at 99.8%), under a JEOL JMS-HX110 (JEOL Ltd., Tokyo, Japan) Spectrum for identification.

(3) Territrem B Quantitative Analysis

Filtrate the methanol solution through a 0.45 μm film and use HPLC (Pomp Model 576, Gaskuro Kygyo Inc., Tokyo, Japan) to analyze the territrem B content. The specifications were as follows: Vercopak Intersil 5 ODS column (4.6 × 250 mm), the mobile phase of acetonnitrile: methanol: water was at the ratio of 6: 4, speed at 1.0 mL/min, detective wave length of 335 mm (Spectro Detector 502U, Gaskuro Kogyo Inc., Tokyo, Japan).

(4) Territrem B Calibration Curve

Added methanol to the territrem B standard till the density reach 1mg/ml, then dilute it with methanol to make solutions with different density at 0, 20, 50, 80, 150, 200, and 250 ng/L. Use the peak size as the Y axis and the solution density as the X axis to generate the territrem B quantitative linear regression equation and the regression coefficient.

(IV) *Evaluate territrem B inter- and intra-day testings and set the HPLC detection limit density*

Equation three types of territrem B with density of 50, 100 and 200 ng/ml, and perform inter-day analysis five times under HPLC analysis conditions. At the same time, perform analysis for five consecutive days to acquire the following precision and accuracy equations : Precision = (S.D of concentration / mean of concentration) x 100; Accuracy = [(mean calculated concentration - actual concentration) / actual concentratio] × 100. The HPLC detection limit = 3 x (S.D. of concentration / mean of concentration) x analytic concentration x injection volume.

(V) *Test territrem recovery from rice media*

Take 50, 100 and 200 ng of territrem B and put into the rice media. Process with the above method and analyze the territrem content by HPLC to calculate the recovery rate.

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RESULT AND DISCUSSION

I. Territrem B Qualitative Analysis

The purpose of this analysis was to identify the territrem B produced by rice media from different *A. terreus* strains inoculation with one- and two-dimensional thin layer chromatography. The thin layer chromatography analysis incorporated the previous study methods[17] and changed the benzene : ethylacetate ratio to 1:1. The result showed that the two-dimensional Rf values of territrem B in the comparative groups were 0.55 and 0.64. In the chloroform extraction from the rice media of 17 different strains after 14 days of cultivation, only *A terreus* 32111 and 32644 showed the same Rf value. The blue fluorescent material (Fig. 2) similar to that of territrem B was also found under the ultraviolet light.

II. *Territrem Qualitative Analysis*

Figure 3A was the HPLC chromatograph of the standard territrem B. The figure showed that the territrem appeared after 9.5 minutes under the conditions used. The peak size was 214528. Take the blue fluorescent material acquired from *A. terreus* strain 32111 and 32644, solve with methanol and analyze by HPLC with results shown in Figure 3B and 3C. The peak sizes were 156198 and 168138, respectively. Both figures showed identical retention time peaking at 9.5 minutes. To confirm the peak was the same as that of territrem B standard, the territrem B standard was added into *A terreus* specimen 32111 and 32664. The result showed in addition to the retention time peak, the peak sizes were 270726 and 382666, respectively. From the above result we confirmed that only *A terreus* strains 32111 and 32664 could produce territrem B. Territrem B was not found in the other 15 strains.

III. *Identifying Territrem B*

The territrem B-like material, purified from the rice media of strains 32111 and 32644 (purification at 99.8%), was identified under JOEL JMS-HX110 spectrum. The $[M+1]^+$ was 527, and ion fragments 181, 291, 413 and 493 were the same as those of territrem B[10]. We thus concluded that the material was territrem B.

IV. *Standard curve equation and coefficient of territrem B*

In order to evaluate the accuracy of HPLC quantitative analysis on territrem B, we first established the territrem B standard calibration curve by equationing different density standard solutions at 0, 0.02, 0.05, 0.08, 0.15, 0.20, and 0.25 μg/mL. After repeatedly injecting each solution for three times to conduct regression analysis, the calibration curve of territrem B was derived as shown in Figure 4. Linear regression equation and the coefficients were Y = 1428.5X + 306.0 and 0.9998, respectively, indicating a clear linier relationship.

V. *Evaluate territrem B inter- and intra-day testings and set the HPLC detection limit density*

In addition to establishing the accuracy of HPLC quantitative method on territrem B, precision and sensitivity were also essential to the analysis for a correct content analysis on territrem B. From Table 1, we learned that the standard deviation of the 50, 100, and 200 ng testings that were conducted five times a day for five days was between 1.6%~2.8%, the deviation of intra-day analysis was between 0.6%~2.3%, and the detection limit density was 5-12 ng/mL. We concluded that this method would provide an accurate, precise and sensitive testing results on territrem B.

Table 1. Intra-day and inter-day analytical precision and accuracy data and detection limit of territrem B

	Acutal concentration	Mean of calculated concentration	Precision	Accuracy	Detection limit
	(ng/mL)		R. S. D. (%)		(ng/mL)
Intra-day					
	50	50.3 ± 0.8a	+1.6	+0.5	7.1
	100	100.5 ± 1.6	+1.6	+0.5	4.7
	200	202.3 ± 5.8	+2.8	+1.2	9.5
Inter-day					
	50	50.6 ± 1.0	+1.9	+1.2	8.7
	100	100.5 ± 2.3	+2.3	+0.5	5.8
	200	199.8 ± 1.1	+0.6	-0.1	11.6

a: n=5

Table 2. Recovery rate of territrem B added to rice media

Spiked levels (ng)	Analyzed levels (ng)	Recovery rate (%)	Mean ± S.D. (%)	R.S.D (%)
50	45.7 ± 2.5a	91.5		
100	94.5 ± 4.6	94.5	93.7 ± 2.0	2.1
200	190.5 ± 12.4	95.2		

a: n=3

VI. *Additional recovery test on territrem B*

Extract territrem based on the method from the previous study[16], setting the maximum solution of chloroform in territrem B at 40.62 mg/mL would be better than other solutions such as acetone at 5.77 mg/mL, tetrahydrofuran 4.38 mg/mL, ethylacetate 3.60 mg/mL, benzene 1.88 mg/mL and methanol at 1.45 mg/mL. Therefore, we used chloroform as the eluent of the rice media extracts. From Table 2, we learned that the rice media recovery rate of the three different density TRB was between 91.48%~95.20%, indicating a good recovery performance of the chloroform extracts.

VII. *Test the territrem B content generated by different strains of A terreus*

Table 3 showed the HPLC results for territrem B from 17 strains of *A terreus* inoculated in rice and cultivated for 14 days in 28˚C. From the Table indicated that among the 17 strains of *A terreus*, territrem B was detected in strains 32111 and 32664 content at 0.20 mg/100g rice and 0.31 mg/100g rice, while territrem B was not detected in the rest 15 strains. When *A terreus* 32111 and 32664 contaminated rice, they not only affected the rice quality, but also raised the hazard of food poisoning. Also, strains that produced territrem B were local strains - No. 32111 was from Chunghwa, No. 32664 was from Hsinfong Hsien, as strain No.23-1 was from Hsinchu county. Other strains were from other regions: No. 30135, 31128, 32068, 32652 (a mutated strain of 31128), 32670, 32677, 32678, 32679, 32681, 32688, 32689 and 32800 were from the U.S. A; No. 32877 was from Australia and No. 32680 was from Africa. This confirmed that regional characteristic was connected to the strains that produced territrem B. The result led to a further exploration on the difference of the secondary metabolized enzyme in the synthetic fungus in the strains, and the difference of DNA or mRNA of the strains. The environment also affected the production of the fungus toxin[18], such as carbon, phosphorus, enzyme induction and other factors (such as temperature, humidity, pH level, still or shaking, anaerobe or aerobe). These also require further study.

Table 3. Territem B analysis by TLC and HPLC from various strains of *Aspergillus terreus*

Strain of *Aspergillus terreus*	TLC	HPLC (mg/100g rice)
30135	No[a]	ND[b]
31128	No	ND
32068	No	ND
32111	Yes	0.193 ± 0.005[c]
32652	No	ND
32653	No	ND
32664	Yes	0.310 ± 0.010
32677	No	ND
32678	No	ND
32679	No	ND
32680	No	ND
32681	No	ND
32688	No	ND
32689	No	ND
32800	No	ND
32877	No	ND

a: No blue spot found in comparing to territrem B standard on one-dimensional TLC plate under long-wave UV.

b: ND, Not detected.

c: Data mean ± S. D. of three samples.

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利用層析法探討不同 *Aspergillus terreus* 菌株於米中產生土震素B之能力

方信裕[1]　危貴金[1]　彭福佐[2]*

1. 中華醫事學院食品營養系
2. 台大醫學院毒理學研究所

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摘　要

土震素B（territrem B）為土麴菌（*Aspergillus terreus*）產生之震顫性黴菌毒素，可導致動物全身性顫抖、流涎、抽搐、甚至導致死亡，其作用機制為抑制乙醯膽鹼酵素（acetylcholinesterase）的活性。本研究目的在於探討食品工業發展研究所菌種保存中心之17株*A. terreus*於米基中產生土震素B的能力。首先從薄膜層析法及高效能液相層析法進行分析，發現僅編號32111與32664之菌株可產生與土震素B 類似之藍色螢光物質，進一步將此藍色螢光物質以質譜儀鑑定為土震素B。土震素B的含量以高效能液相層析法配合C_{18}逆向層析管柱及於335 nm波長下測定。土震素B之標準線性公式為Y=1428.5X+306.0（R^2 = 0.9998），其中Y為土震素B於高效能液相層析儀之面積，X為土震素B的濃度，顯示良好的線性關係；同日間及異日間之相對標準偏差分別為1.6～2.8%及0.6～2.3%，顯示具有良好之準確性及精確性；添加回收率在91.5～95.2%之間，顯示氯仿可作為米基萃取液；利用此方法可知*A. terreus* 32111及32664產生土震素B含量分別為0.2及0.3 mg/100g米。從地源性而言，產生土震素B之*A. terreus* 32111及32664兩株菌為台灣本土之菌株，其它15株分別來自不同國家之*A. terreus*，無法產生土震素B。

關鍵詞：土震素，土麴菌，薄膜層析法，高效能液相層析法