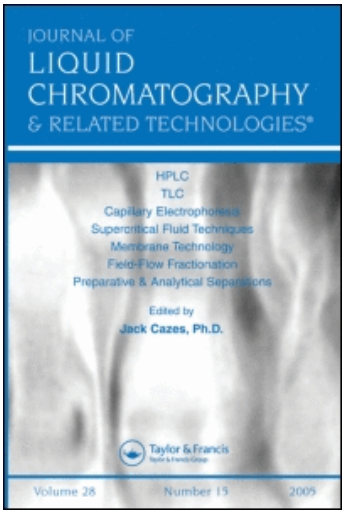

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ISOLATION OF NIVALENOL AND FUSARENON-X FROM PRESSED BARLEY CULTURE BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

A method for the production and isolation of gram quantities of nivalenol and fusarenon-X, trichothecene mycotoxins, is presented. Fusarium graminearum F-1465 was inoculated and cultured on the pressed barley, and the cultured product was extracted with acetonitrile. The crude extracts were subjected to silica gel column chromatography and then to centrifugal partition chromatography (CPC) using two-phase solvent system n-butanol/water and chloroform/methanol/water. The simple procedures were advantageous for the large scale purification of two toxins from cultured pressed barley medium because it was more time and yield effective. Starting from 1 Kg of the pressed barley substrate, 0.34 g of crystalline nivalenol (purity 103 %) was obtained by recrystallization of CPC fraction from hot methanol and 0.78 g of fusarenon-X (purity 77 %) was obtained directly from CPC fraction by evaporating to dryness.

INTRODUCTION

Nivalenol and its monoacetyl precursor, fusarenon-X, are classified as trichothecene mycotoxins having a epoxy group at C-12, C-13 position. Crops are widely contaminated with nivalenol, as well as with deoxynivalenol, T-2 toxin and diacetoxyscirpenol. These mycotoxins are produced by various species of Fusarium and their toxicological behavior or metabolism in mammals have been investigated, except nivalenol and fusarenon-X. Few studies on toxicity and metabolism of nivalenol and fusarenon-X have been published because it has been difficult to isolate sufficient amounts of these pure toxins for toxicological evaluation and for analytical standards.

Various purification methods for trichothecene mycotoxins have been reported, including preparative thin layer chromatography (TLC)(1,2,3), combination of column chromatographies (CC)(4,5,6), and preparative liquid chromatography (LC)(7,8). However, these methods were not sufficient to obtain large ammounts of toxin, and rapidly. Besides a little sample loading capacity, column chromatography technique often causes loss of toxins due to the irreversible adsorption of toxins to column packing materials.

In the present study, we describe that the centrifugal partition chromatography (CPC) can be applied to gram quantities of isolation of nivalenol and fusarenon-X in high purity.

MATERIALS

Fungus Strain

Fusarium graminearum F-1465 was supplied by Dr. Masakatsu Ichinoe, of National Institute of Hygienic Sciences. The strain was pre-cultured on the potato-dextrose agar slant for 10 days at 25 °C before inoculation on the pressed barley substrate.

METHODS

Culture

The pressed barley substrate (500 g per one cultivation) was immersed in water for 1 h and riddled. The autoclaved pressed barley substrate was extended over the stainless steel tray (30 x 30 cm) with a net at the bottom and was inoculated with the fungus strain. Culture was continued for two weeks at 25 ℃.

Extraction and Purification

The culture medium (500 g) was homogenized with 2 liters of acetonitrile by POLYTRON (KINEMATICA, Switzerland). The homogenate was filtered and the residual cake was re-extracted with 1.5 liters of acetonitrile.

The combined filtrate was evaporated to dryness and the residue was dissolved in 150 ml of methanol. Silica gel (150 g, 100 - 200 mesh) was added to methanol solution and the slurry was evaporated to dryness by rotary evaporator. The resulting silica gel powder was layered on the top of glass column (5 cm i.d.) packed with 100 g of silica gel with chloroform and eluted with 1 liter of chloroform/methanol (3:2, V/V). And then evaporated residue was separated by CPC.

Centrifugal partition chromatographs, Sanki Engineering (Kyoto) CPC Model LLI equipped with disk-type partition cell rotor (net volume; 7 liter) and CPC Model LLN equipped with twelve 1000E type partition cell cartridges (net volume; 850 ml) were used for fractionation and purification of toxins.

The partially purified toxins by silica gel column chromatography was dissolved in two-phase solvent system of n-butanol/water and fractionated by CPC Model LLI using the same two-phase solvent system under the following condition, rotor speed; 400 rpm, flow rate; 50 ml/min, detection; ab-

sorbance at 254 nm, operating temperature; room temperature. Upper layer of two-phase solvent system of n-butanol/water was used as stationary phase and the system was developed with 15 liters of bottom phase (reversed descending elution mode). The eluate was collected in 100-ml fractions and toxins in each fraction was detected by TLC of silica gel. Nivalenol and fusarenon-X were eluted between fraction volumes 1-5 liters and 5-15 liters, respectively.

For further purification, the crude nivalenol and fusarenon-X fractions obtained by the first CPC were fractionated by CPC Model LLN, using two-phase solvent system of chloroform/methanol/water (65:65:40, V/V) under the following condition, rotor speed; 800 rpm, flow rate; 6 ml/min, detection; absorbanse at 254 nm, operating temperature; 20 °C. The crude nivalenol fraction was evaporated to dryness and was dissolved in the two-phase solvent system (30 ml of upper layer and 20 ml of lower layer). The solution was fractionated by CPC under normal descending elution mode using the upper layer as stationary phase. After 1.9 liters development by lower layer, the solvent flow was switched to the reversed ascending elution mode using the upper layer as mobile phase. The eluate (1 liter) was collected in 100-ml fractions, in which toxins were also detected by TLC. Nivalenol fraction from 450 to 650 ml were collected and evaporated to dryness. The residue was dissolved in 10 ml of hot methanol and recrystallized by standing at -10 °C overnight. The precipitated crystals were filtered, washed with cold methanol, and dried in a desiccator. The crude fusarenon-X fraction obtained by the first CPC was dissolved in two phase solvent system (30 ml of upper layer and 20 ml of lower layer) and purified by the second CPC. After developing with 1.3 liters of upper layer under reversed ascending mode, the elution mode was switched from reversed ascending to normal descending using the lower layer as mobile phase. The eluate (900 ml) was collected in 9-ml fractions followed by monitoring toxins by TLC. Fusarenon-X containing fractions (from 450 to 720 ml) were collected and evaporated to dryness to form the crytalline powder.

Assay and Identification

Nivalenol and fusarenon-X obtained by CPC were detected by TLC using pre-coated silica gel plates (HPTLC plate, size; 10 x 10 cm, gel thickness; 250 μm, E. Merck, Darmstadt). One μl of the eluate was applied on a plate and was developed with chloroform/methanol (7:1, V/V). Toxins on plate were visualized as blue fluorescent spots under 365 nm irradiation by spraying aluminum chloride solution followed by heating on the hot plate at 130 °C .

The contents of two toxins in the culture medium and purity of the isolated toxins were determined by gas chromatography as their TMS derivatives by reacting with trimethylchlorosilane/trimethylsilylimidazole/ethylacetate (0.2:1:9,V/V) for 15 min at room temperature. Gas chromatograph; Hewlett-Packard 5890A (^{63}Ni ECD). Column; HP Ultra-2 capillary column (25 m x 0.2 mm i.d.). Carrier gas; helium (column head pressure 150 KPa). Split ratio; 1/60. Column oven temperature; hold at 150 °C (1 min.), programmed from 150 to 270 °C at 40 °C /min and 270 to 300 °C at 4 °C /min.

Mass spectra were measured at 30 eV ionization voltage by direct insertion method using JOEL-DX300 mass spectrometer. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were measured with tetramethylsilane as an internal standard in acetone d_6 by JOEL JNM-GX400 NMR spectrometer.

RESULTS AND DISCUSSION

Partition coefficients of nivalenol and fusarenon-X in various two-phase solvent systems were determined and summarized in Table 1. Chroroform/methanol/water (65:65:40, v/v) system and n-butanol/water system could be suitable rather than ethylacetate/water system for separation of toxins by CPC. In this study, n-butanol/water system was used for the first CPC, in which large ammounts of sample was subjected to CPC, and chloroform/methanol/water system was used further purification by CPC.

TABLE 1

Comparison of Partition Coefficients of Nivalenol and Fusarenon-X in Various Solvent Systems

Solvent system	Nivalenol	Fusarenon-X
$CHCl_3$/MeOH/H_2O (65:65:40)	3.26	0.266
n-BuOH/H_2O (satd)	0.660	2.59
EtOAc/H_2O (satd)	0.0794	1.94

Measured at 25 °C.
Partition coefficient; K(upper/lower).

From 1 Kg (500 g x 2) of pressed barley culture, 90 g of crude extract was obtained. After passing through silica gel column to remove polar materials, preliminary fractionation by CPC LLI system was carried out, giving 3.87 g of nivalenol fraction and 3.78 g of fusarenon-X fraction. Each crude fraction was further purified by CPC Model LLN system. Finally, 0.34 g of cryatalline nivalenol and 0.78 g of crystalline fusarenon-X were obtained. A summary of the purification procedure is shown in Table 2. Final yields of nivalenol and fusarenon-X calculated from their contents in the starting acetonitrile extract were 44 and 68 %, respectively. Fig. 1 shows CPC elution profiles of partially purified extract. Since peak resolution of nivalenol from impurities was incomplete, a part of the peak (elution volumes 450-600 ml) was collected and recrystallized from hot methanol, giving 0.34 g of pure crystals of nivalenol. Fusarenon-X seems to be well resolved from impurities on the chromatogram, so that the most of the peak fractions (elution volumes 450-720 ml) was evaporated to dryness; yield 68 % and purity 77 % without recrystallization.

Mass spectrum and assignment of NMR spectra of nivalenol are shown in Fig. 2 and Table 3.

TABLE 2

Isolation Procedure of Nivalenol and Fusarenon-X from Pressed Barley Culture

Procedure step	Ammount, g (recovery, %)		
	Total materials	Nivalenol	Fusarenon-X
Extraction	90 (100)	0.773 (100)	0.881 (100)
Silica gel CC	24 (27)	0.772 (100)	0.877 (100)
CPC-LLI			
Nivalenol fr.	3.87 (4.3)	-*	
Fusarenon-X fr.	3.78 (4.2)		-*
CPC-LLN			
Nivalenol fr.	0.75 (0.8)	-*	0
Fusarenon-X fr.	0.78 (0.9)	0	0.60 (68)
Recryst.			
Nivalenol fr.	0.33 (0.4)	0.34 (44)	

* not determined

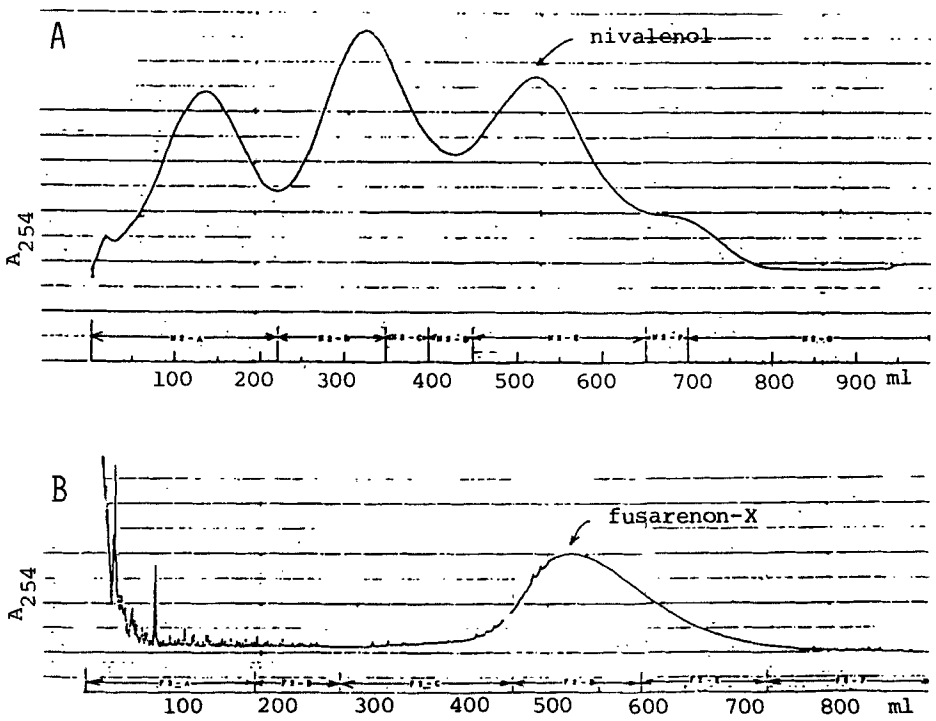

FIGURE 1. CPC profiles of nivalenol (A) and fusarenon-X (B) fractions extracted from pressed barley culture as described in the text, monitoring absorbance at 254 nm.

TABLE 3

^{13}C (100 MHz) and ^{1}H (400 MHz) Chemical Shift* Assignment for Nivalenol

Carbon no.	^{13}C Chemical shift	^{1}H Chemical shift
2	81.19	3.53 ($J_{2,3}$=5.0)
3	81.13	4.16 ($J_{3,2}$=5.0; $J_{3,4}$=5.0 $J_{3,OH}$=4.0)
3 OH		4.48
4	81.03	4.55 ($J_{4,3}$=5.0; $J_{4,OH}$=3.5)
4 OH		3.85
5	45.14	
6	50.20	
7	74.93	4.82 ($J_{7,OH}$=2.7)
7 OH		4.03
8	200.65	
9	135.79	
10	139.65	6.57 ($J_{10,11}$=6.0; $J_{10,16}$=1.5)
11	70.25	4.72 ($J_{11,10}$=6.0)
12	65.65	
13	45.70	2.93 (J_{AB}=4.5)
		2.90
14	8.24	1.08 (singlet)
15	61.63	3.84 (J_{AB}=11.5; $J_{15,OH}$=5.0)
		3.74
15 OH		3.76
16	15.23	1.79 ($J_{16,10}$=1.5; $J_{16,11}$=1.0)

* ppm from tetramethylsilane.

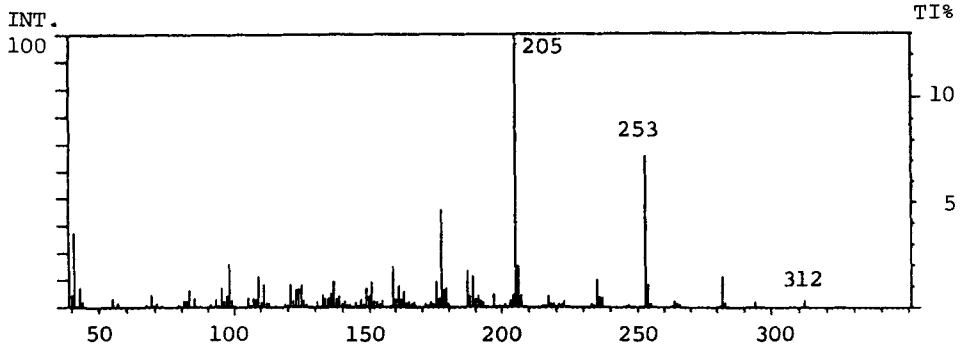

FIGURE 2. Electron impact mass spectrum of nivalenol.

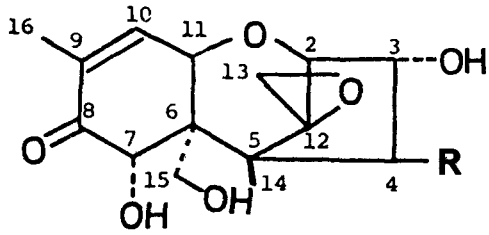

FIGURE 3. Structure of nivalenol and fusarenon-X.

The compound derived from fusarenon-X by alkalline hydrolysis showed the same mass spectrum as that of nivalenol. Moreover, these compounds isolated in this study have the same retention time as that of each authentic standard in capillary gas chromatogram. From these results the isolated compounds were identified as nivalenol and fusarenon-X having molecular structure shown in Fig. 3.

Silica gel column chromatography followed by florisil column chromatography according to Lee's method (5) resulted in a very low recovery because of irreversible adsorption of the toxins to silica gel. Similar phenomena has been reported by Ehrlich et. al.(6) in purification of deoxynivalenol. Our method showed minimum amount of sample loss of toxins during the procedure. The purification by CPC is better than other purification methods for these toxins from the following point of view; high recovery (approximately 100 %), rapid preparation, and large sample loading capacity. CPC system was useful for the purpose of large scale separation of pure toxins.

From the results described in this report we could obtain sufficient amounts of the toxins, nivalenol and fusarenon-X, for succeeding of toxicological and metabolic studies.

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