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An Improved Method for the Purification of the Trichothecene Deoxynivalenol (Vomitoxin) from *Fusarium graminearum* Culture

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It was hypothesized that a simplified and efficient strategy could be developed for large-scale production and purification of the mycotoxin deoxynivalenol from *Fusarium graminearum* rice cultures for toxicological studies. *F. graminearum* R6576 was cultured on rice and extracted with methanol, and the extract was concentrated and subjected to silica gel low-pressure liquid chromatography (LPLC) under a hexane−acetone gradient system. Deoxynivalenol isolation was monitored by thin-layer chromatography, and fractions containing deoxynivalenol were pooled, concentrated, and applied to a second LPLC column under the same conditions. An enriched deoxynivalenol fraction was obtained, which yielded a crystalline material. Repeated crystallization yielded spectroscopically pure deoxynivalenol. The identity of this compound was confirmed by HPLC comparison to an authentic deoxynivalenol standard, FABMS analysis, and comparison of the ^{1}H and ^{13}C NMR spectra with published data. This simplified purification scheme eliminated many laborious steps and equipment previously required to obtain gram quantities of crystalline deoxynivalenol for biological testing in animal models.

KEYWORDS: Mycotoxin; trichothecene; deoxynivalenol; vomitoxin; *Fusarium graminearum*; purification

INTRODUCTION

Deoxynivalenol (3,7,15-trihydroxy-12,13-epoxytricothec-9-en-8-one) (**Figure 1**) was first characterized and named following its isolation from *Fusarium*-infected barley in Japan (*1*). Vesonder et al. (*2*) concurrently isolated the same compound from *Fusarium*-infected corn in the United States and named it "vomitoxin" because of its capacity to induce emesis in swine. Since deoxynivalenol can be produced during fungal colonization of grains such as wheat, barley, and corn in the field and during storage (*3*−*5*), the potential for human exposure and illness exists (*6*, *7*). The known toxicity of deoxynivalenol (*8*), combined with its worldwide prevalence, makes further safety evaluation a priority.

Thus, there is a need for large quantities of pure deoxynivalenol from *Fusarium* cultures for additional in vitro and in vivo toxicological studies. Currently, no sufficient synthetic method exists for the production of deoxynivalenol (*9*). Previous work demonstrated that solid substrate cultures are superior to liquid cultures for the production of deoxynivalenol (*10*, *11*). Methods that have been described for the large-scale production of crystalline deoxynivalenol are labor intensive and lack efficiency due to multiple purification procedures (*11*−*13*). A methodology is needed for laboratories possessing minimal equipment and lacking large fermentors for the production of large quantities of deoxynivalenol, particularly for those laboratories not specialized in either microbiology or chemical isolation.

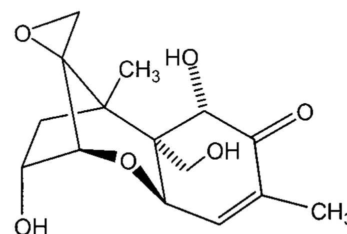

Figure 1. Structure of the mycotoxin deoxynivalenol.

In previous work (*11*), we identified a high deoxynivalenol-yielding strain of *Fusarium graminearum* as well as the optimal conditions with regard to substrate, water activity, culture vessel, temperature, atmosphere, and incubation time. We hypothesized here that the efficiency for downstream purification could be greatly improved by using a simple low-pressure liquid chromatography (LPLC) system coupled with crystallization. The overall protocol eliminates the need for liquid fermentations, complex chromatographic separations, and multiple partitioning

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steps, allowing crystalline deoxynivalenol to be easily and consistently obtained in a timely manner.

MATERIALS AND METHODS

General Experimental Procedures. Low-pressure liquid chromatography was performed using a 37-mm-i.d. glass chromatography column and precolumn (Ace Glass, Inc., Vineland, NJ), dry packed with 70−230 mesh silica (Sigma, St. Louis, MO), and an RP-SY-ICSC pump (Fluid Metering, Inc., Oyster Bay, NY) coupled to a pulse dampener. A Retriever III fraction collector (ISCO, Lincoln, NE) was used to collect fractions for the LPLC. An authentic deoxynivalenol standard, used for thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), was purchased from Sigma, while deoxynivalenol, used for crystallization, was kindly supplied by Dr. Robert Eppley (Food and Drug Administration, Washington, DC). Silica TLC plates (20 × 20 cm^2, 250 μm) for deoxynivalenol identification in LPLC fractions were obtained from Fisher (Pittsburgh, PA). Plates were developed in toluene−ethyl acetate (2:3), sprayed with 15% aluminum chloride (15 g $AlCl_3 \cdot 6H_2O$ in 85 mL of ethanol and 15 mL of H_2O), and heated for 5 min at 110 °C (*11*). Deoxynivalenol was visualized under UV (λ 365 nm) light and identified by comparison to a commercial standard.

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian Inova 300-MHz spectrometer (Palo Alto, CA). FABMS analysis was performed at the Michigan State University Mass Spectrometry Facility using a JEOL HX-110 double-focusing mass spectrometer (Peabody, MA) operating in the positive ion mode. High-performance liquid chromatography was performed with a model 2300 HPLC pump equipped with a 22-cm × 4.6-mm-i.d. RP-18 Spheri-10 column and a 3-cm × 4.6-mm-i.d. guard cartridge (Brownlee Lab, Inc., Santa Clara, CA), coupled to a variable-wavelength absorbance detector (ISCO) at λ 210 nm, using a mobile phase of 20% methanol (2 mL/min).

Preparation of Rice Cultures. Previous studies comparing deoxynivalenol production in experimentally infected corn and wheat with a number of *F. graminearum* isolates demonstrated that *F. graminearum* R6575 was a high-yielding strain for the preparative-scale production of deoxynivalenol (*11*, *14*−*16*). This fungus was used to inoculate potato dextrose agar plates as previously described (*11*). Following incubation at 25 °C in the dark for 7 d, 4-mm agar plugs of the red mycelial colonies were removed and added to 100 mL of sterile carboxymethylcellulose media (15 g of carboxymethylcellulose, 1 L of H_2O, 0.5 g of $NH_4NO_3 \cdot 7H_2O$, and 1.0 g of yeast extract) (*11*) in a 500-mL flask. Cultures were incubated for 3−5 d at 25 °C with agitation on a rotary shaker and filtered through sterile cheesecloth. Macroconidia were enumerated using a hemacytometer.

Following previously described procedures (*11*), 350 g of enriched white rice and 150 mL distilled water were autoclaved in 2.8-L Fernbach flasks stoppered with foam plugs, with 16 flasks used per batch. Flasks were inoculated with 10^6 macroconidia and incubated at 28 °C in the dark for 18 d. At the end of the incubation period, deoxynivalenol was extracted from the rice cultures and purified.

Extraction of Rice Cultures. Upon completion of the incubation period, 1.4 L of 70% (v/v) methanol was added to each flask, and the rice mass at the bottom of the flask was broken into pieces with a glass rod. Each flask was extracted by soaking the rice in methanol for 15 h, after which the methanol was decanted and passed through cheesecloth. Cultures were further extracted for an additional 2 h by adding 250 mL of 100% methanol to each flask. All extracts were pooled and filtered (Whatman No. 4), and the methanol was evaporated over a steam bath until the extract was reduced to approximately one-fourth of its original volume. The extract was cooled, saturated with NaCl, and allowed to stand for 18 h before filtration (Whatman No. 4) to remove the precipitate.

Following evaporation of the methanol, the resulting extract was divided into 500-mL portions and extracted six times with 1 L of ethyl acetate using a separatory funnel. The ethyl acetate was evaporated in vacuo, producing a viscous, oily brown extract (7 g).

Low-Pressure Liquid Chromatography. The ethyl acetate extract (3.5 g) was dissolved in 4:1 hexane−acetone (10 mL) and applied to a silica gel LPLC precolumn. The column (equilibrated with 4:1 hexane−acetone) was sequentially eluted with 4:1 (500 mL), 7:3 (1 L), 3:2 (1 L), 1:1 (500 mL), 2:3 (500 mL), and 1:3 (500 mL) hexane−acetone, followed by 250 mL of acetone. Twelve-milliliter fractions were collected, and every fifth fraction was analyzed by TLC to determine the presence of deoxynivalenol. LPLC was repeated with the remaining ethyl acetate extract, and the deoxynivalenol-containing fractions of both runs were combined, concentrated in vacuo (2.7 g), dissolved in 7:3 hexane−acetone, and loaded on the precolumn for further LPLC separation under the conditions previously described, except with the modification of starting the gradient with 7:3 hexane−acetone. Twelve-milliliter fractions were collected and analyzed by TLC. Fractions containing deoxynivalenol were combined and concentrated in vacuo.

Crystallization of deoxynivalenol was achieved by dissolving the extract in a minimum amount of methanol. Due to the presence of an oily yellow contaminant that coeluted with deoxynivalenol, seed crystals of pure deoxynivalenol were added to induce crystallization. The solution was held at 4 °C until crystals formed, and the viscous, oily, deep yellow mother liquor was removed by decanting. Crystallization was repeated a second time in the same manner as the first crystallization. Following the completion of the second crystallization, the pale yellow mother liquor was removed by decanting, and the crystals were dissolved in a minimum amount of methanol. The third crystallization was performed without the addition of seed crystals by holding the solution at 4 °C until crystallization was complete (yield, 2.01 g; 72% recovery).

Confirmation of Deoxynivalenol Structure and Determination of Purity. The ^1H and ^{13}C NMR spectra of deoxynivalenol isolated from rice culture were identical to those previously reported (*17*, *18*). The FABMS analysis also confirmed the identity of deoxynivalenol, yielding a molecular ion m/z 297.2 ($[M + H]^+$). High-performance liquid chromatography was used to assess the purity of the crystalline deoxynivalenol. Purity was determined by comparing the peak areas of several deoxynivalenol samples isolated from rice culture to a standard curve generated using a commercial deoxynivalenol standard.

RESULTS AND DISCUSSION

Crystalline deoxynivalenol was readily obtained using the new method. The purity of the deoxynivalenol isolated from rice culture was determined to be >99% on the basis of HPLC analysis and comparison to a commercial standard. In three additional trials of this new method, approximately 2 g of high-purity, crystalline deoxynivalenol was consistently produced per 16-flask batch (350 g of rice per flask), or 357 mg/kg. This yield was higher than reported previously (*11*−*14*).

Previous methods required that cultures be blended (*11*−*13*) to extract as much deoxynivalenol as possible; however, this process increases dissolution of rice components into the initial matrix, thus making it more difficult to purify the deoxynivalenol. A second issue with blending is safety relative to equipment contamination and aerosolization of mycotoxin. Foregoing the blending and instead passing the 70% methanol extract through cheesecloth and filtering the extract saved time while achieving a high final yield of deoxynivalenol. Additionally, increasing the percentage of methanol used for extraction from 60% (*11*) to 70% reduced the time required to evaporate the solvent on the steam bath.

Low- or medium-pressure silica gel chromatography readily facilitated the purification of large quantities of deoxynivalenol. The use of LPLC with a hexane−acetone gradient, coupled with a fraction collector, effectively separated deoxynivalenol with ease. TLC screening for deoxynivalenol was rapid, and the use of hexane−acetone to elute deoxynivalenol allowed rapid evaporation of the solvent from the extract. The new method using LPLC effectively eliminated the need for repeated partitioning (water−ethyl acetate or water−methylene chloride), charcoal−alumina columns (*11*), Florisil columns (*13*), or

Sephadex LH-20 columns (*11*) used in previous methods. Additional cleanup prior to crystallization was not necessary as with former methods (*11*, *13*), saving time in the purification.

Crystallization was achieved by using seed crystals of deoxynivalenol. The highly viscous, oily yellow impurity that coeluted with deoxynivalenol from the LPLC column was readily soluble in ice-cold methanol, acetone, and chloroform. In an effort to preserve as much deoxynivalenol as possible, the oily mother liquor was removed by decanting before crystallization was repeated. Crystallization did not require close attention, unlike other means of additional purification (*11*, *13*). In addition, the mother liquor was saved for subsequent purification in the next LPLC. Repeated crystallization proved to be a rapid means of obtaining high-purity deoxynivalenol.

In summary, crystalline deoxynivalenol was obtained as a result of the development of a simplified method to obtain gram quantities of deoxynivalenol for use in toxicological studies. Silica gel LPLC using a hexane−acetone solvent gradient produced deoxynivalenol that was sufficiently pure for crystallization. Repeated crystallization yielded >99% pure deoxynivalenol by HPLC analysis. This methodology for the purification of deoxynivalenol from rice culture may be performed in most laboratories with little need for additional equipment customarily employed by microbiology laboratories, such as liquid fermentors. Compared to previously published methods, our improved, simplified method facilitated the rapid production of large quantities of high-quality, crystalline deoxynivalenol in a time- and cost-efficient manner.

LITERATURE CITED

(1) Morooka, N.; Uratsuji, N.; Yoshizawa, T.; Yamamoto, H. Studies on the toxic effects of barley infected with *Fusarium* spp. *J. Food Hygiene Soc. Jpn*. **1972**, *13*, 368−375.

(2) Vesonder, R. F.; Ciegler, A.; Jensen, A. H. Isolation of the emetic principle from *Fusarium*-infected corn. *Appl. Microbiol*. **1973**, *26*, 1008−1010.

(3) Placinta, C. M.; D'Mello, J. P. F.; Macdonald, A. M. C. A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Anim. Feed. Sci. Technol*. **1999**, *78*, 21−37.

(4) Abouzied, M. M.; Azcona, J. I.; Braselton, W. E.; Pestka, J. J. Immunochemical assessment of mycotoxins in 1989 grain foods: Evidence for deoxynivalenol (vomitoxin) contamination. *Appl. Environ. Microbiol*. **1991**, *57*, 672−677.

(5) Tanaka, T.; Hasegawa, A.; Yamamoto, S.; Lee, U. S.; Sugiura, Y.; Ueno, Y. Worldwide contamination of cereals by the *Fusarium* toxins nivalenol, deoxynivalenol and zearalenone. Survey of 19 countries. *J. Agric. Food Chem*. **1988**, *36*, 979−983.

(6) Luo, X. Y. Outbreaks of moldy cereals poisoning in China. *Issues in Food Safety*; Toxicology Forum, Inc.: Washington, DC, 1988; pp 56−63.

(7) Bhat, R.; Beedu, S. R.; Ramkirishna, Y.; Munshi, K. L. Outbreak of tricothecene mycotoxicosis associated with consumption of mould-damaged wheat products in Kashmir Valley, India. *Lancet* **1989**, *1*, 35−37.

(8) Rotter, B. A.; Prelusky, D. B.; Pestka, J. J. Toxicology of deoxynivalenol (vomitoxin). *J. Toxicol. Environ. Health* **1996**, *48*, 1−34.

(9) Colvin, E. W.; Thom, I. G. A synthetic approach to the trichothecene deoxynivalenol. *Tetrahedron* **1986**, *42*, 3137−3146.

(10) Pestka, J. J.; El-Bahrawy, A.; Hart, L. P. Deoxynivalenol and 15-monoacetyl deoxynivalenol production by *Fusarium graminearum* R6576 in liquid media. *Mycopathologia* **1985**, *92*, 23−28.

(11) Witt, M. F.; Hart, L. P.; Pestka, J. J. Purification of deoxynivalenol (vomitoxin) by water-saturated silica gel chromatography. *J. Agric. Food Chem*. **1985**, *33*, 745−748.

(12) Scott, P. M.; Lawrence, G. A.; Telli, A.; Iyengar, J. R. Preparation of deoxynivalenol (vomitoxin) from field-inoculated corn. *J. Assoc. Off. Anal. Chem*. **1984**, *67*, 32−34.

(13) Ehrlich, K. C.; Lillehoj, E. B. Simple method for isolation of 4-deoxynivalenol from rice inoculated with *Fusarium graminearum*. *Appl. Environ. Microbiol*. **1984**, *48*, 1053−1054.

(14) Hart, L. P.; Brasselton, W. E.; Stebbins, T. C. Production of zearalenone and deoxynivalenol in commercial sweet corn. *Plant Dis*. **1982**, *66*, 1133−1135.

(15) Hart, L. P.; Pestka, J. J.; Liu, M. T. Effect of kernel development and wet periods on production of deoxynivalenol in wheat infected with *Gibberella zeae*. *Phytopathology* **1984**, *74*, 1415−1418.

(16) El-Bahrawy, A.; Hart, L. P.; Pestka, J. J. Comparison of deoxynivalenol (vomitoxin) production by *Fusarium graminearum* isolates in corn steep-supplemented Fries medium. *J. Food Prot.* **1985**, *48*, 705−708.

(17) Savard, M. E.; Blackwell, B. A. Spectral characteristics of secondary metabolites from *Fusarium* fungi. In *Mycotoxins in Grain*−*Compounds other than Aflatoxin*; Miller, J. D., Trenholm, H. L., Eds.; Eagon Press: St. Paul, MN, 1994; pp 138−139.

(18) Cole, R. J.; Cox, R. H. The tricothecenes. *Handbook of toxic fungal metabolites*; Academic Press: New York, 1981; pp 152−263.

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