APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1978, p. 408-411 Vol. 36, No. 3
0099-2240/78/0036-0408$02.00/0

Printed in U.S.A.

Production and Analysis of Citrinin in Corn

L. K. JACKSON AND A. CIEGLER*

Northern Regional Research Center, Federal Research, Science and Education Administration, U.S. Department of Agriculture, Peoria, Illinois 61604

Received for publication 20 June 1978

A convenient method for the production and analysis of citrinin in corn is described. Up to 2.964 g of citrinin can be produced by *Penicillium citrinum* per kg of corn by harvesting on day 21 or later. The analysis method has a lower detection limit of 0.25 ppm. Heating citrinin-contaminated corn destroys citrinin but may produce another toxin instead.

Citrinin, a toxic secondary metabolite of certain fungi, was first isolated in 1931 (6) from *Penicillium citrinum* Thom. It has been implicated in porcine nephropathy (9) and has been found as a natural contaminant of corn, rice, wheat, rye, barley, and oats (12, 13, 15, 16). Thus, citrinin is a potentially important mycotoxin that may be ingested by humans and other animals and could cause chronic disease (5).

To date, no methods have been reported for the production and analysis of citrinin in corn; most production methods utilize liquid media (4, 6, 14, 17). The fact that Scott et al. (16) have found as much as 0.08 g of citrinin per kg of naturally contaminated wheat suggests that grains could possibly be a good substrate for citrinin production. Damodaran et al. (3) have published the only solid substrate production of citrinin, but they report only 0.08 g of citrinin per kg of rice and 0.0024 g/kg of wheat. They also report a colorimetric analysis method for citrinin in rice or wheat which is not as sensitive or convenient as quantitative thin-layer chromatography (TLC) of the fluorescent citrinin. Hald and Krogh (5) and Scott et al. (16) developed analytical methods similar to the one described herein, but these methods were not tested on corn.

This paper reports a method for the production of up to 2.964 g of citrinin per kg of corn. The analysis of corn contaminated with at least 0.25 ppm of citrinin is discussed. The effect of heat on citrinin and citrinin-contaminated corn was explored, and a possible new toxin was isolated from heated citrinin-contaminated corn.

MATERIALS AND METHODS

Production and isolation of citrinin on corn. *P. citrinum* NRRL 5927 and 5907 were stored at 4 to 5°C on Blakeslee malt agar, which contains 2% malt extract, 0.1% peptone, 2% dextrose, and 2% agar. *P. citrinum* NRRL 5927, which is the same as AUA532, and NRRL 5907 were both isolated from coastal Bermuda grass by U. L. Diener of Auburn University. Cracked corn was moistened with 333 ml of water per kg of corn and autoclaved for 20 min at 121°C. After cooling, the clumps of corn were broken, and 300 g was added per Fernbach flask and autoclaved again for 20 min at 121°C. The corn was inoculated with a spore suspension of *P. citrinum* NRRL 5907 or 5927 and incubated at 25°C as stationary cultures. After 21 days, 1,250 ml of chloroform was added to each Fernbach flask and allowed to soak overnight to kill the spores. To the chloroform solution, 125 ml of water and 50 ml of concentrated HCl were added and allowed to stand for 30 min. The suspension was blended for 5 min and filtered, and the chloroform layer was retained. The chloroform was rinsed with water and extracted with 0.1 M $NaHCO_3$. The $NaHCO_3$ solution was acidified with concentrated HCl to pH 2.5 and extracted with chloroform, this latter solvent extract then being concentrated to 50 ml. The chloroform solution was washed with water and again extracted with 0.1 M $NaHCO_3$. The aqueous layer was acidified to pH 2.5, and citrinin was collected by filtration. The citrinin was dissolved in $CHCl_3$ and dried with Na_2SO_4. The citrinin readily crystallized from hot absolute ethanol, and yields were determined by weight.

Citrinin was monitored by comparison with an authentic sample by using TLC on silica gel F254, $CHCl_3$-MeOH, 75:25. The toxin was detected by fluorescence under a 366-nm UV light. Comparison of the chemical and physical characteristics of the crystalline material with UV, infrared, mass, and nuclear magnetic resonance spectra and the melting point of authentic citrinin as reported in the literature (8, 10, 11, 13, 17) confirmed the identification of the compound.

Citrinin production curve and analysis. *P. citrinum* NRRL 5927 was utilized for the citrinin production curve, and the citrinin was isolated as described previously except that 100 g of corn per 500-ml Erlenmeyer flask was used for each analysis. Two flasks were analyzed after 3, 7, 14, 21, and 28 days. The chloroform extract of citrinin was partitioned into $NaHCO_3$ once, the aqueous layer was acidified, and the acidic layer was extracted with chloroform. This chloroform extract was dried in a rotary evaporator, taken up in 1 ml of $CHCl_3$, and analyzed by quantitative TLC. The TLC was described previously, but in

addition, the amount of citrinin was determined by visual comparisons of fluorescing zones of sample with known quantities of standard citrinin spotted on the same plate. The standard (100 μg/ml in chloroform) and sample were spotted at 1, 2, 3, 5, and 10 μl, and the sample was diluted when necessary.

Spiking experiments. One-hundred-gram amounts of citrinin-free, cracked corn were spiked at various levels with a standard chloroform solution of citrinin. The same standard solution was used for spiking and quantitative TLC.

Heat degradation of citrinin. Citrinin was prepared as explained in the production section, but prior to citrinin isolation, the spores on the corn were killed by various heat treatments instead of a room temperature sterilization with chloroform. The fermentation was autoclaved for 20 min at 121°C, steamed for 15 min at 100°C, or steamed for 5 min at 100°C and then dried for 3 h at 50°C.

A standard 1-mg/ml citrinin solution in water was also heat treated by autoclaving for 20 min at 121°C. An attempt to recover the citrinin was then made by the method described above.

Production and isolation of the unidentified toxin. The toxin was produced and isolated from heat-treated corn as described previously for citrinin, except that the toxin remained in the chloroform layer during $NaHCO_3$ extraction. The chloroform was repeatedly extracted with 0.1 M $NaCHO_3$ until no citrinin could be detected by TLC.

An attempt was also made to isolate the toxin from fermentations on corn or yeast extract sucrose medium which were not heat treated prior to toxin isolation. Five-hundred milliliters of 2% yeast extract and 4% sucrose was inoculated with *P. citrinum* NRRL 5927 and incubated for 14 days at 28°C as stationary cultures. The mycelium and liquid were acidified to pH 1 to 2 with HCl, and 500 ml of chloroform was added. The mixture was blended for 5 min on low speed and filtered through cheesecloth. The chloroform layer was removed, and the aqueous layer was extracted again with chloroform. The combined chloroform extracts were washed with water and extracted with 0.1 M $NaHCO_3$, and the citrinin was obtained from the $NaHCO_3$ layer as before. The chloroform layer was concentrated and exhaustively extracted with $NaHCO_3$ until no citrinin could be detected by TLC; the chloroform layer was examined for the crude toxin.

The crude toxin was assayed by injecting mice with various fractions from the extraction procedure. The fractions were dried and taken up in 1 ml of cottonseed oil, and 0.2 ml of the solution was injected intraperitoneally. The fraction which caused deaths was considered to contain the unknown toxin, and it was found only in the chloroform layer of heat-treated fermented corn. Further purification of this fraction is in progress.

RESULTS AND DISCUSSION

W. T. Roberts supplied us with corn fermented with *P. citrinum* AUA 532, which failed to produce citrinin. In the process of determining why this particular fermentation did not produce citrinin, it was found that corn actually is an excellent substrate for citrinin production. Harvesting on day 14 produced good yields of citrinin: 1.06, 0.90, and 0.84 mg/g of corn from trials 3, 6, and 7 (Table 1). Even higher yields were obtained by harvesting on day 21 or later (Table 2). These yields were considerably higher than those previously reported by Damodaran et al. (3) (0.08 mg/g of rice and 0.0024 mg/g of wheat). *P. citrinum* NRRL 5927 or 5907 produced nearly equivalent amounts of citrinin, as seen in trials 6 and 7 of Table 1. Although many liquid substrate methods for citrinin production suggest

TABLE 1. *Citrinin yield and toxicity of unknown toxin with different substrates and sterilization procedures*

Trial	Substrate and inoculum	Method of fermentation sterilization prior to citrinin isolation	Crystalline citrinin yield,[a] harvested at day 14	Presence of unknown toxin
1[b]	Corn, NRRL 5927	Dried overnight, 70°C	Oily, noncrystalline solids	+
2[b]	Corn, NRRL 5927	Dried overnight, 70°C	Oily, noncrystalline solids	+
3	Corn, NRRL 5927	$CHCl_3$, no heat	1.06 mg/g of corn	−
4	Corn, NRRL 5927	Autoclaved, dried for 3 h, 50°C	0.12 mg/g (oily, noncrystalline solid)	+
5	Corn, NRRL 5927	Steamed for 15 min, dried for 3 h, 50°C	0.12 mg/g	+
6	Corn, NRRL 5927	Steamed for 5 min, dried for 3 h, 50°C	0.90 mg/g	+
7[c]	Corn, NRRL 5907	Steamed for 5 min, dried for 3 h, 50°C	0.84 mg/g	−
8[d]	YES, NRRL 5927	$CHCl_3$, no heat	1.19 g/liter	−
9[d]	YES, NRRL 5927	$CHCl_3$, no heat	0.805 g/liter	−

[a] Yield by weight of first crystallization.
[b] Fermented by W. T. Roberts and supplied to us for analysis.
[c] NRRL 5907 was tested because it is generally a good citrinin producer.
[d] YES is 2% yeast extract and 4% sucrose.

TABLE 2. *Production of citrinin on corn by P. citrinum NRRL 5927*

Time (days)	Citrinin (mg/g of corn)[a]	Avg citrinin (mg/g of corn)
3	0.000524	0.001307
3	0.001566	
7	1.044	0.870
7	0.696	
14	2.470	2.223
14	1.976	
21	2.964	2.964
21	2.964	
28	2.964	2.964
28	2.964	

[a] Yield by quantitative TLC.

precipitation of citrinin from the medium with acid, in practice we found (Table 1, trials 8 and 9) that very little citrinin actually precipitated. Better citrinin recoveries were obtained by our procedure, and the toxin obtained was purer and more readily crystallized.

Before a citrinin production curve could be determined, a method was needed to analyze the small amounts of citrinin that would be produced initially in the fermentation. Using the extraction and quantitative TLC system mentioned in Materials and Methods, 100% recoveries of citrinin were obtained from a standard aqueous solution of citrinin. When corn was spiked at various levels of citrinin between 5 and 0.1 ppm, a lower detection limit of 0.25 ppm was obtained. Recovery levels from corn spiked between 5 and 0.25 ppm ranged from 32 to 13%, with lower recoveries for lower spiking levels. Although these recoveries are low, Stubblefield (unpublished data) and Wilson et al. (18) both report recoveries in this range, and Scott et al. (16) report that lower recoveries are obtained if extraction and TLC cannot be done on the same day.

To determine the best time to harvest the citrinin for maximum yield, a production curve was determined, and results are shown in Table 2. Harvesting on day 21 or later produced the best yield, 2.964 mg/g of corn. The discrepancy between Tables 1 and 2 on yield at day 14 probably occurs because yields in Table 1 were obtained by actual weight of crystalline material and yields in Table 2 were obtained by quantitative TLC. Probably more citrinin was produced than was actually obtained as crystalline material due to losses during crystallization.

A fermentation that was briefly sterilized by steam prior to extraction resulted in good yields of citrinin (Table 1, trials 6 and 7); however, very little citrinin was isolated when fermented corn was accidentally steamed too long (Table 1, trial 5). This suggested that citrinin was probably heat sensitive. This fact was confirmed by varying the sterilization procedure for the fermented corn. The maximum citrinin yield was obtained with no heat, the minimum yield was obtained with autoclaving, and intermediate yields were obtained with intermediate heat conditions. It appears that molded corn supplied to us by W. T. Roberts (trials 1 and 2) failed to yield citrinin because it was degraded during the drying procedure. Therefore, even heating the fermented corn at 70°C for an extended period of time resulted in the loss of citrinin. These findings are in agreement with Neely et al. (13) and Kawashiro et al. (7), who found a decrease in absorbance of standard citrinin solutions beginning at 60 to 70°C.

The heat sensitivity of citrinin was further confirmed when less than 19% of the citrinin could be recovered from an autoclaved aqueous solution of citrinin. Quantitative TLC was used to determine the percent recovery, and it also showed that additional substances were now present. When the standard solution was not autoclaved, 100% of the citrinin was readily recovered, and TLC indicated that only citrinin was present.

Thus, the experimenter should avoid prolonged heating of fermentations prior to citrinin extraction or during analysis of citrinin. This may appear to contradict Chu's (1) statement that citrinin is stable to autoclaving, but what his paper actually notes is that autoclaving does not cause loss of antibiotic power. Citrinin could thus be degraded by autoclaving and the degradation products still retain antibiotic capacity.

Even though no citrinin could be detected in the fermented corn supplied to us by W. T. Roberts, the corn was still toxic, so a toxin other than citrinin was probably present. It was explained previously that failure to detect citrinin in this fermentation was due to its degradation during the drying procedure, so it would be unlikely that the toxicity would be due to substrate-bound citrinin which could not be isolated by the extraction method that was used. By injecting mice with various fractions from the extraction procedure, a toxin was found in the chloroform extracts after the citrinin was removed by $NaHCO_3$ extraction. The toxin was not citrinin, because the chloroform was repeatedly extracted with $NaHCO_3$ until no citrinin could be detected by TLC. Further purification of the chloroform extract failed to yield a pure toxin; but during citrinin production trials, as can be seen by examination of Table 1, the unknown toxin was produced by NRRL 5927 only when the fermentation was exposed to heat.

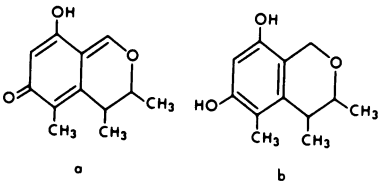

FIG. 1. *(a) Decarboxycitrinin; (b) decarboxydihydrocitrinin.*

Thus, it appears that heat degrades citrinin, but toxicity is retained in a fraction that is chloroform soluble and $NaCHO_3$ insoluble. This would agree with Chu's (1) findings that antibiotic power is retained after autoclaving.

A simple explanation for the appearance of the unknown toxin during heating is that the toxin is a degradation product of citrinin. Thus, an aqueous solution of citrinin was degraded in the autoclave, and the chloroform solubles that were not removed by $NaHCO_3$ were examined for chemical composition and toxicity. Because the toxin was not removed by $NaHCO_3$ like citrinin, it seemed probable that the heat had caused decarboxylation of the citrinin to a compound such as decarboxycitrinin (Fig. 1a), which is a natural metabolite of *P. citrinum* (2), or decarboxydihydrocitrinin (Fig. 1b). An infrared spectrum of the chloroform-soluble degradation products lacked a carbonyl, which would favor a decarboxylation product and would rule out decarboxycitrinin in favor of decarboxydihydrocitrinin. However, when the chloroform-soluble degradation products of citrinin were injected in mice, no toxicity was found. Thus, it seems that the appearance of a new toxin upon heating citrinin-contaminated corn is not simply a citrinin degradation product. Also, as can be seen by comparing trials 6 and 7 of Table 1, NRRL 5907, which is a citrinin producer, failed to yield a toxin. The toxicity may be due to an interaction of the degraded citrinin and the corn upon which it is grown, or the toxicity may be due to the degradation of another *P. citrinum* NRRL 5927 metabolite. Additional research is needed to clarify why NRRL 5907 did not produce a toxin and whether the toxin is a citrinin degradation product, a *P. citrinum* degradation product, or some sort of adduct of a degradation product and substrate.

ACKNOWLEDGMENTS

We are grateful to W. T. Roberts for pointing out to us the problem associated with fermenting corn with *P. citrinin* and for supplying us initially with molded corn samples. We also thank D. Weisleder for the nuclear magnetic resonance spectrum and W. K. Rohwedder for the mass spectrum.

LITERATURE CITED

1. **Chu, W.** 1946. Miscellaneous pharmacological actions of citrinin. J. Lab. Clin. Med. **331**:72-78.
2. **Curtis, R. F., C. H. Hassall, and M. Nazar.** 1968. The biosynthesis of phenols. XV. Some metabolites of *Penicillium citrinum* related to citrinin. J. Chem. Soc. C, p. 85-93.
3. **Damodaran, C., C. S. Ramadoss, and E. R. B. Shanmugasundaram.** 1973. A rapid procedure for the isolation, identification and estimation of citrinin. Anal. Biochem. **52**:482-488.
4. **Davis, N. D., U. L. Diener, and G. A. Sansing.** 1975. Medium-scale production of citrinin by *Penicillium citrinum* in a semisynthetic medium. Appl. Environ. Microbiol. **29**:118-120.
5. **Hald, B., and P. Krogh.** 1973. Analysis and chemical confirmation of citrinin in barley. J. Assoc. Off. Anal. Chem. **56**:1440-1443.
6. **Hetherington, A. C., and H. Raistrick.** 1931. Studies in the biochemistry of micro-organisms. XI. On the production and chemical constitution of a new yellow colouring matter, citrinin, produced from glucose by *Penicillium citrinum* Thom. Philos. Trans. R. Soc. London Ser. B **220**:269-297.
7. **Kawashiro, I., H. Tanabe, H. Takeuchi, and C. Nishimura.** 1955. Determination of citrinin contained in rice by fluorometry. Bull. Natl. Inst. Hyg. Sci. (Tokyo) **73**:191-196 (from Chem. Abstr. **50**:7229b, 1956).
8. **Kovac, S., P. Nemec, V. Betina, and J. Balan.** 1961. Chemical structure of citrinin. Nature (London) **190**:1104-1105.
9. **Krogh, P., E. Hasselager, and P. Friis.** 1970. Studies on fungal nephrotoxicity. 2. Isolation of two nephrotoxic compounds from *Penicillium viridicatum* Westling: citrinin and oxalic acid. Acta Pathol. Microbiol. Scand. **78**:401-413.
10. **Mathieson, D. W., and W. B. Whalley.** 1964. The chemistry of fungi. XLIV. The conformation of citrinin. J. Chem. Soc., p. 4640-4641.
11. **Mirchink, T. G., V. S. Blagoveshchenskii, and V. A. Fedorov.** 1967. Identification of the toxin formed by *Penicillium citrinum* no. 19 and study of its herbicidal properties. Microbiology **36**:866-869.
12. **Mislivec, P. B., and J. Tuite.** 1970. Species of *Penicillium* occurring in freshly-harvested and in stored dent corn kernels. Mycologia **62**:67-74.
13. **Neely, W. C., S. P. Ellis, N. D. Davis, and U. L. Diener.** 1972. Spectroanalytical parameters of fungal metabolites. I. Citrinin. J. Assoc. Off. Anal. Chem. **55**:1122-1127.
14. **Rodig, O. R., L. C. Ellis, and I. T. Glover.** 1966. The biosynthesis of citrinin in *Penicillium citrinum*. I. Production and degradation of citrinin. Biochemistry **5**:2451-2458.
15. **Saito, M., M. Enomota, and T. Tatsuno.** 1971. Yellowed rice toxins—citrinin, p. 357-367. *In* A. Ciegler, S. Kadis, and S. J. Ajl (ed.), Microbial toxins, vol. 6. Academic Press Inc., New York.
16. **Scott, P. M., W. Van Walbeek, B. Kennedy, and D. Anyeti.** 1972. Mycotoxins (ochratoxin A, citrinin, and sterigmatocystin) and toxigenic fungi in grains and other agricultural products. J. Agric. Food Chem. **20**:1103-1109.
17. **Timonin, M. I., and J. W. Rouatt.** 1944. Production of citrinin by *Aspergillus* sp. of the *Candidus* group. Can. J. Public Health **35**:80-88.
18. **Wilson, D. M., W. H. Tabor, and M. W. Trucksess.** 1976. Screening method for the detection of aflatoxin, ochratoxin, zearalenone, penicillic acid, and citrinin. J. Assoc. Off. Anal. Chem. **59**:125-127.