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FLUORODENSITOMETRIC ASSAY OF PENICILLIC ACID

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SUMMARY

A fluorodensitometric method was developed for the quantitative analysis of penicillic acid, a toxic secondary mold metabolite. The method depends on the conversion by ammonia fumes of penicillic acid after thin-layer chromatography to a fluorescent derivative. This technic was used successfully to analyze moldy corn and a liquid fermentation for this toxin.

INTRODUCTION

Penicillic acid, a secondary metabolite first isolated from *Penicillium puberulum*[1], has since been shown to be produced by a variety of fungi. The compound possesses antimicrobial activity[2,3], is toxic to mammals[4], and has proved carcinogenic to rats[5]. KURTZMAN AND CIEGLER[6] found that in blue-eye-diseased corn caused by *Penicillium martensii*, large quantities of penicillic acid are produced when high-moisture corn is stored at low temperatures (5 to 10°). Since blue-dye disease may be common in combine harvested corn, penicillic acid represents a potential mycotoxin hazard to humans and livestock. The colorimetric methods to detect this compound[7,8] lack the sensitivity required for a mycotoxin assay, the smallest detectable amount being 200 µg. We found that the product of the reaction between penicillic acid and ammonia fluoresced an intense blue and that this property could be used as the basis for a sensitive fluorodensitometric assay.

MATERIALS AND METHODS

Penicillic acid

The compound was produced fermentatively with *Penicillium cyclopium* NRRL 1888. Penicillic acid was recovered by the method of BENTLEY AND KEIL[8] except for two final recrystallizations from benzene to remove traces of a yellow pigment; the crystals were then sublimed under vacuum at 80–90°. The final product did not de-

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press the melting point of authentic penicillic acid (84.2 to 84.8° as determined with a Mettler FPI* melting point apparatus).

To produce the ammoniated derivative, an acetone solution of penicillic acid was streaked onto a preparative plate of Brinkmann Silica Gel GHR and developed in ether. The plate, after evaporation of solvent, was exposed for 5 min to concentrated ammonia fumes. The resulting bright blue fluorescing band was eluted with methanol and its spectral characteristics were determined in an Aminco-Bowman spectrophotofluorometer; excitation was at 350 mμ and emission at 440 mμ (Fig. 1).

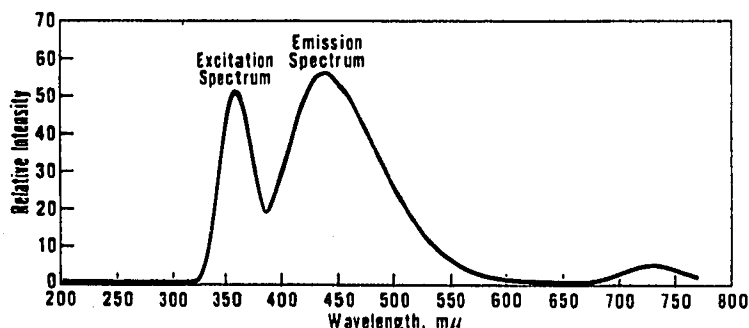

Fig. 1. Excitation and emission spectrum of the ammonia–penicillic acid reaction derivative in methanol.

TLC conditions

Standard 20 × 20 cm plates were coated with 250 μ of Silica Gel GHR. Various amounts of a standard penicillic acid solution in benzene (1 μg/μl) were spotted about 2 cm apart and 2.5 cm from the bottom of the plate. The plate was developed in chloroform–ethyl acetate–formic acid (60:40:1) in an unlined and unequilibrated Desaga–Brinkmann glass tank. After the solvent front had traveled about 16 cm, the plate was removed, dried under a gentle warm air stream from a hand dryer until formic acid could no longer be detected, and then exposed to concentrated ammonia fumes in a lined tank for 3 min. The R_F value of penicillic acid in the solvent system used was 0.45. Other solvent systems that can be used are as follows: chloroform–methanol (97:3), R_F value 0.5; chloroform–acetone (93:7), R_F value 0.1; chloroform–ethanol (90:10), R_F value 0.75; chloroform–ethyl acetate (50:50), R_F value 0.6.

Fluorodensitometric assay

About 1–2 cm of silica gel was removed from the bottom and top of the developed plate and small aluminum rails slipped along the cleared sides. The plate was placed on an automatic scanning stage of a Photovolt Model 530 densitometer. The stage contained a search unit (Model 52-C) equipped with a UV light source (320 to 390 mμ), a primary filter (365 mμ), a secondary filter (445 mμ), and a primary slit (0.1 by 15 mm). A Beckman 10-in. recorder with an automatic integrator was used to quantitate the assay. The distance between the TLC plate and the search unit was adjusted to 1 mm.

After the developed plate was placed face down on the stage, the densitometer was manually adjusted to the center of a standard zone containing 7 μg penicillic

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acid. The response given to this amount was arbitrarily set at 70 % on the recorder with dilutions of unknowns being adjusted to read out between 10 and 90 %. The densitometer was also set to the lowest background at which no fluorescence occurred to obtain a base line. The fluorescent zones were manually centered inside the 15-mm slit of the search unit so that the path of development would be scanned. The stage was set so that the fluorescent zone was located near the origin of the plate, and the plate scanned automatically toward the solvent front. All analyses were carried out in a darkened room to prevent erratic readings of the densitometer.

A standard curve was prepared for each analysis by obtaining the area from the integration units from two different amounts of toxin, usually 3 and 7 μg, spotted in duplicate. The concentration of penicillic acid in unknowns was then determined by calculating the number of integration units from each of two samples analyzed in duplicate thus giving four readings per experimental condition; values were obtained by direct reading off the standard curve. The following formula was used to calculate the concentration of penicillic acid in an unknown where a solid substrate, such as corn, was analyzed: μg of penicillic acid per g of substrate $= (U_g/U_v)\ (V_D \cdot V_E)/W_x$, where U_g = weight of toxin (μg) determined from the standard curve; U_v = volume of solution spotted (μl); V_D = dilution of the solution spotted; V_E = volume of solvent (μl) used for the original extraction; W_x = weight of solids (g) extracted.

If an unknown solution was analyzed, the concentration of toxin in μg/ml was obtained readily from: $(U_g/U_v) \times 10^3$.

Extraction of solids

Generally, 50 g of molded grain, *e.g.* corn, was extracted with 250 ml of chloroform–methanol (90:10) for 3 min in a Waring Blendor. The extract was filtered through anhydrous soldium sulfate and the first 50 ml were collected for analysis.

RESULTS AND DISCUSSION

Standard assay

The densitometric assay using the procedure developed followed Beer's law between about 1 to 9 μg penicillic acid. A typical standard curve is shown in Fig. 2.

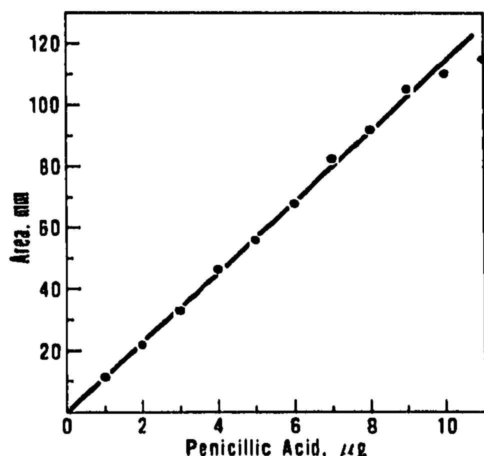

Fig. 2. Standard fluorodensitometric assay of penicillic acid TLC on Silica Gel GHR using chloroform–ethyl acetate–formic acid (60:40:1) for development; developed plate exposed to ammonia fumes for 3 min and fluorescence measured densitometrically.

At higher quantities some quenching occurred. Quenching could be partially avoided by eliminating formic acid from the TLC solvent system; a more diffuse spot results. However, the spot occasionally diffused too much so that it overlapped the slit of the search unit. The average deviation in values usually ranged from 3 to 7% with the largest deviation occurring with the smaller amounts spotted, *i.e.*, 1 to 3 μl, probably because of the difficulty in accurately dispensing small volumes of solution. This variation is similar to that noted for the fluorodensitometric analysis of aflatoxin[9]. Although the concentration of the unknown can be determined by visual comparison to a spotted series of standards, our experience with aflatoxin assays indicates a potential accuracy no greater than $\pm 17\%$ (ref. 10).

In the densitometric tracings, base-line variation caused by varying silica gel thickness across the plate was negligible. Even-thickness plates were more readily obtained by blending the Silica Gel GHR (30 g)–water (64 ml) mixture in a Waring Blendor for 30 sec before spreading. Commercial plates with a plastic backing were unsatisfactory because they passed too much light in areas lacking the fluorescent zones and because they were too flexible.

Ammonia exposure time

Increasing the time of plate exposure to ammonia from 3 to 5, 20, or 40 min only slightly increased fluorescence from the various amounts of toxin present as indicated by the increased slope of the dose-response curve, from 1.7 to 1.9 in one experiment. However, the final concentration of a given unknown was not affected since its fluorescence intensity also increased proportionately. After a 40-min exposure, the erratic data obtained indicated some degradation of the toxin.

Stability of fluorescence

No change in data was noted if the plates were stored 4 h after exposure to ammonia; this indicates relative compound stability. However, 24 h later, there was considerable loss of fluorescence and none could be detected at the lower amounts of acid (1–3 μg). Consequently, it would be advisable to assay the plates reasonably soon after preparative procedures are completed.

The identity of the fluorescent derivative is not known. An analogous reaction has not been reported in the literature. Reaction of penicillic acid with NaOH converts the toxin to its nonfluorescent tautomer, γ-keto-β-methoxy-δ-methylene-Δ^{α}-hexenoic acid. Hence, the reaction with ammonia to give a fluorescent derivative does not involve a simple alkaline hydrolysis of the lactone ring. In addition, no fluorescence results from ammoniation of the keto tautomer. Structural studies are now in progress.

Assay of moldy corn and a liquid fermentation

Whole corn that had been inoculated with *P. martensii* NRRL 3612 was incubated at 15° and analyzed periodically for penicillic acid content (Fig. 3). High concentrations of lipid did not interfere with the assay; hence, a cleanup procedure was not required.

Data in Table I indicate that only an average of 80% of the toxin was removed on the first extraction. However, the concentration of toxin in the first solvent extract probably represents an accurate value for the amount of toxin in the entire sample

since an average of only 84% of the solvent is recovered after blending, the remainder being occluded in the residual gummy solids.

Penicillic acid was also produced by fermentation with *P. cyclopium* NRRL 1888 in static liquid culture[8]. The supernatant was sampled directly (2 to 10 μl spotted for TLC) and no difficulties were encountered in the analyses (Fig. 3).

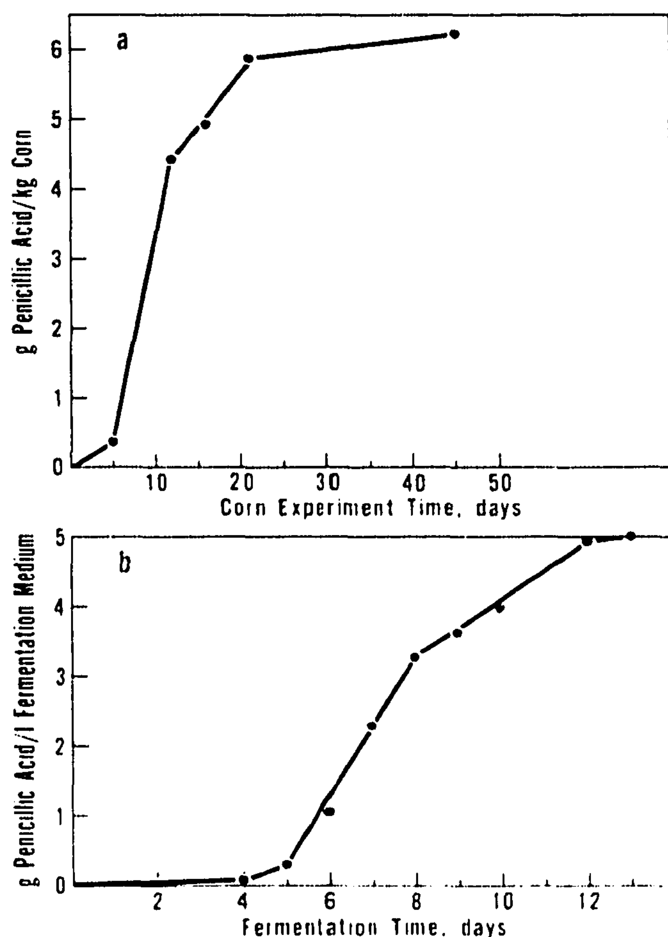

Fig. 3. Production of penicillic acid in corn and by liquid fermentation. Solid culture: 50 g corn inoculated with *Penicillium martensii*, incubated at 15°. Liquid culture: *Penicillium cyclopium* at 25°, static fermentation; supernatant analyzed.

TABLE I

ANALYSIS OF PENICILLIC ACID FROM MOLDY CORN

Sample[a]	*First extraction*[b] *(g toxin/kg corn)*	*Volume solvent recovered* (%)	*Second extraction*[b] *(g toxin/kg corn)*	*Total toxin* (g)	*First recovery* (%)
1	15.0	76	3.6	18.6	81
2	17.0	83	2.3	19.3	88
3	14.7	79	3.0	17.7	83
Av.	15.6	79.3	3.0	18.5	84

[a] Each sample contained 50 g corn molded with *Penicillium martensii* NRRL 3612 for 109 days at 5°.

[b] Each extraction used 250 ml of chloroform–methanol (90:10).

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