

WATER-SOLUBLE PRODUCTS FROM PATULIN DURING ALCOHOLIC FERMENTATION OF APPLE JUICE

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ABSTRACT

During the course of alcoholic fermentation, 50 ppm of ^{14}C -patulin added to apple juice was converted to other materials. The products were largely nonvolatile and water soluble. Little, if any, patulin was metabolized to CO_2 . Comparison of the thin-layer chromatographic (TLC) migration of the major patulin products from fermentation with the products of the reaction of patulin with cysteine indicated that at least 58% of the added patulin was converted to substances other than adducts of cysteine, peptides and proteins. In addition to a major TLC-immobile component (21%), at least six TLC-mobile patulin products were observed.

INTRODUCTION

PATULIN is a mycotoxin reported to produce tumors in rats at the point of injection when administered subcutaneously (Dickens and Jones, 1961). Patulin is produced in high yields by a number of food-contaminating fungi (Enomoto and Saito, 1972). One of these fungi, *Penicillium expansum*, is an important cause of storage rot of apples, and patulin has been isolated from infected apples and commercial apple juice (Harwig et al., 1973a; Stoloff, 1975). The fate of patulin during alcoholic fermentation has not been resolved. French workers have reported the presence of residual patulin following fermentation of apple juice (Drilleau and Bohuon, 1973), but others have reported that patulin "disappears" or is substantially reduced during fermentation of apple juice (Burroughs, 1977; Harwig et al., 1973b; Stinson et al., 1978).

This study was undertaken to determine whether patulin per se persists following alcoholic fermentation of apple juice and to determine the nature of other products that may arise from patulin during fermentation.

EXPERIMENTAL

Materials

Uniformly labeled ^{14}C -patulin was prepared by an unpublished modification of the fermentation method of Norstadt and McCalla (1969) that we developed. Dr. Norstadt supplied a freshly isolated strain of *Penicillium urticae*. The fungus produced the highest yields of patulin when the medium (potato dextrose broth) was autoclaved long enough to give a golden yellow color (121°C for 30-45 min). Uniformly labeled ^{14}C -glucose was added after autoclaving to serve as the radioactive precursor. Monitoring the fermentation by pH measurement rather than elapsed time was crucial for favorable yields. Two successive physiological phases of *P. urticae* growth control patulin biosynthesis (Bu'Lock et al., 1965). When this medium is used, the pH increases slowly from 5.2 to 6.7 during the first phase and dips slightly to 6.3 during the second. Maximum patulin concentration was attained at the end of the second phase, which was detected by the dip in the pH. It was important to harvest patulin at this point as autolysis immediately ensued (marked by increasing pH) and rapidly destroyed patulin. Peak patulin concentration was variable, but 1000 mg/liter after 10 days was representative, falling to 600 mg/liter at 12 days. When the pH started to rise

after reaching a minimum, the mycelial growth was removed by filtering through cheesecloth. The fermentation broth was concentrated in vacuo and extracted with ethyl acetate. The extract was dried over MgSO_4 , concentrated to a thick sirup, and placed on a silicic acid column, and the patulin was eluted with benzene/chloroform, 3:1. The solvent was removed under vacuum, and the patulin was purified by successive recrystallizations from benzene and ether. Minimal heating was used to avoid the decomposition during recrystallization that has been reported (Katzman et al., 1944). Identity and purity of the labeled patulin were established by comparing its IR spectrum and migration on thin layer chromatographic (TLC) plates with those of authentic patulin supplied by R. D. Stubblefield (NRRC, USDA, Peoria, IL) and by running a mixed melting point with authentic patulin. The specific activity of the purified material was 0.00355 mCi/mmol as measured by the procedure described later. All of the ^{14}C activity was associated with the patulin TLC spot. Other procedures for preparing ^{14}C -patulin have been reported (Lovett, 1972; Dailey et al., 1977; Nip and Chu, 1977).

Patulin-cysteine adduct was prepared by reacting patulin with cysteine in aqueous solution in 1:1 ratio at 0.0065M concentrations. Base (0.1N NaOH) was added as required to maintain the pH at 3.5, the approximate pH of apple juice. After the solution had stood for 2 wk at room temperature, the characteristic UV absorption peak for patulin at 275 nm diminished to approximately 50%. No new UV peaks were observed. Patulin, but not cysteine, could be detected by TLC. The solution became yellow and a small quantity of yellow precipitate developed, corresponding to the precipitate described by Ciegler et al. (1976). Patulin-cysteine adduct solution was concentrated by evaporation under nitrogen prior to application to TLC plates.

Fermentation procedure

Standard fermentation practices were followed. A commercial "Champagne" yeast, *Saccharomyces cerevisiae* v. *ellipsoides* (Universal Foods, Red Star Yeast Division, Milwaukee, WI; originally from Institute Pasteur, Paris, France) was obtained as a lyophilized powder in an aluminum foil package labeled as sufficient for 5 gal. This was suspended in 100 ml water, and 0.5 ml was used for inoculation. A stock solution was prepared from whole, pasteurized apple juice by adding sugar to 22°B . Other components were 50 ppm ^{14}C -patulin, 0.08% $(\text{NH}_4)_2\text{SO}_4$, and 20 ppm Dowex Anti-Foam A (conveniently added after being absorbed on sugar). A 125-ml aliquot of this solution was placed in a 125 ml Erlenmeyer flask (actual capacity approx 140 ml) and inoculated with the yeast suspension. The flask was covered with a one hole rubber stopper in which a glass tube had been inserted. The gas evolved by the fermentation was bubbled successively through three solutions: the standard fermentation gas lock in which the gas is bubbled through water to insure anaerobic conditions, then two solutions of aqueous KOH to absorb the CO_2 formed by the fermentation. As evolution of gas slowed in the latter part of the fermentation, it was necessary to raise the inlet tube of the second flask above the liquid level to prevent backup of liquid into the fermentation gas lock.

The fermentation was allowed to proceed 28 days at 21°C , until gentle swirling of the flask produced no further gas evolution, and then allowed to stand two additional days to permit the yeast to form a compact sediment.

A control experiment consisted of adding ^{14}C -patulin to unfermented, pasteurized apple juice containing 20% added ethanol to prevent microbial growth. This solution was analyzed for patulin and ^{14}C -patulin products after standing for 30 days at 21°C .

Detection of radioactivity

Radioactive events were detected by a Nuclear-Chicago Mark IV liquid scintillation counter (G.D. Searle & Co., Des Plaines, IL), and Aquasol, a liquid scintillation solvent containing a thixotropic material (Nuclear-Chicago). This formed a stiff gel upon addition of sufficient water. Liquid materials could be analyzed when added to the

solvent. Solid materials were analyzed after being suspended in stiff thixotropic gels.

TLC procedures

Uniplate silica gel GF 250 μ plates (Analtech) and preabsorbant LQF 250 μ plates (Kontes) were used. The organic phase of a mixture of n-butanol/acetic acid/water (10:1:3) was used as the developing solvent. Zones on the TLC plates were detected by UV quenching, ninhydrin, I₂ vapor, and ¹⁴C counting of material scraped from the plates.

Patulin analysis

Patulin was quantitatively analyzed by the method of Stinson et al. (1977).

RESULTS & DISCUSSION

THE ALCOHOLIC FERMENTATION yielded a fermented cider with a typical, pleasant aroma. The pH of the initial apple juice was 3.5, and that of the fermented cider was 4.0. The final Brix was -0.5°. Analysis of the fermented cider for patulin gave negative results by the Stinson et al. (1977) procedure.

The opalescent, fermented cider was clarified to a brilliant clear liquid by centrifugation for 40 min at 20,000g in a refrigerated centrifuge. The sediment from this centrifugation was combined with the original sediment for ¹⁴C analysis. The solutions from the fermentation and the sediment were analyzed for ¹⁴C (Table 1). The original juice before fermentation contained 2,540 cpm/ml.

The fractions of ¹⁴C labeled material consisted of 91.6% water-soluble material that remained in the fermentation flask, 2.9% in the sediment, and 2.0% in the CO₂ traps. The occurrence of the sizeable ¹⁴C fraction, 4.1%, in the fermentation lock was not expected. There was no reason to assume that foaming had occurred, since no tendency was observed during the laboratory working day. A possible explanation could be mechanical entrapment of microscopic droplets during effervescence of the CO₂ bubbles, as approximately 6.5 liters of CO₂ resulted from the fermentation. The finding that the CO₂ traps contained only 2.0% of the ¹⁴C indicated that little, if any, patulin was metabolized to yield CO₂.

Attempts to cleanly separate by TLC the soluble, ¹⁴C-containing materials in clarified, fermented cider were unsuccessful. After the plates were developed and dried, the disposition of ¹⁴C was 21% at or near the origin from R_f 0.00–0.08, 16% from R_f 0.08–0.66, 11% from R_f 0.66–0.75, and 47% from R_f 0.75–1.00. Five percent of the applied activity was not recovered from dried TLC plates and was assumed lost in the form of volatile compounds; 95% was present in relatively nonvolatile compounds which persisted on TLC plates even after prolonged drying. Components on TLC plates at R_f's 0.00–0.08 and 0.66–0.75 were visualized by either UV light (254 nm) or treatment with iodine vapor; ninhydrin-positive components were observed only at R_f 0.00–0.08. Since a fermented apple juice control (unspiked with ¹⁴C-patulin and containing no detectable patulin prior to fermentation) also yielded similar components that could be visualized with UV light, iodine vapor, or ninhydrin, the actual ¹⁴C-containing products may not be the visible components.

In the unfermented control, over 98% of the 50 ppm added ¹⁴C-patulin was recovered unchanged at the end of 30 days; only 0.6 ppm was converted to nonvolatile substances that were insoluble in ethyl acetate. This agrees with published data (Scott and Somers, 1968). Preliminary work indicated that the products arising from patulin in unfermented apple juice consisted of at least six components; however, these components existed at such comparatively low concentrations in fermented juice that they could not be detected in the presence of the other products resulting from patulin. Thus, the reaction of patulin with

Table 1—Distribution of ¹⁴C after fermentation of apple juice containing ¹⁴C—Patulin

Fraction	Total cpm	% of original
Clarified fermented cider (118 ml)	289,400	91.6
Sediment from settling and centrifuging 1.6g, ~2 ml	9,200	2.9
Fermentation lock (H ₂ O; 130 ml)	12,800	4.1
First CO ₂ trap (10% KOH; 300 ml)	6,200	2.0
Second CO ₂ trap (10% KOH; 15 ml)	165	0.05
	317,765	100.65

material initially present in unfermented apple juice could account for only 1.2% of the patulin converted to other substances during fermentation.

The reaction of patulin with sulfhydryl-containing amino acids and proteins to form uncharacterized adducts has been advanced to explain the disappearance of patulin in many foods, including apple juice (Ciegler et al., 1976). The sulfhydryl content of apple juice has been reported as less than 0.003 mmole per 100 ml (Scott and Somers, 1968). In a bimolecular reaction, this could account, at most, for the loss of only 4.6 ppm patulin, whereas fermentation resulted in the loss of 50 ppm patulin. To determine whether the formation of adducts with compounds containing sulfhydryl groups could account for the disappearance of patulin, we subjected the patulin-cysteine adduct preparation to TLC analysis under the same conditions as the fermentation product. The most intense UV quenching band occurred at the origin. A number of mobile bands extended up as far as R_f = 0.40, with the most prominent bands at R_f 0.14–0.19 and 0.32–0.40. The lack of mobility of the adduct in comparison to the bulk of the ¹⁴C-containing material from the fermentation indicated that much of the patulin was converted to substances other than adducts of cysteine under the circumstances of this experiment. Adducts of patulin and peptides, such as glutathione, or proteins containing sulfhydryl groups would exhibit even less TLC mobility.

It has been reported that yeast does not produce extracellular materials that can react with patulin (Harwig et al., 1973b). If this is the case, the formation of new ¹⁴C-containing materials from ¹⁴C-patulin during fermentation must result from the metabolic activity of yeast cells on apple juice substrates and/or patulin, itself.

Further work is required to determine the chemical composition and toxicologic properties of the products that arise from patulin during alcoholic fermentation. Thus far, efforts here and elsewhere (Ciegler et al., 1976) to chemically characterize products of patulin degradation or reaction have been unsuccessful because of the apparent instability of the compounds. Ciegler et al. (1976) demonstrated that patulin-cysteine adducts retain teratogenic activity in chick embryo tests. It is possible that other products that may arise during alcoholic fermentation also are biologically active, and it would be unwise to assume that the "disappearance" of patulin per se in any food system nullifies the potential health hazard posed by its initial presence.

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