

Study of Conditions for Production of Roquefortine and Other Metabolites of *Penicillium roqueforti*

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Experiments to determine optimum yields of roquefortine, isofumigaclavine A, and PR toxin, metabolites from *Penicillium roqueforti* Thom, were performed. Four strains, isolated from blue cheese, and five liquid media were evaluated, although not all permutations were studied. Sucrose (15%)-yeast extract (2%) was the medium chosen for time-course studies at 25 and 15°C using one favorable strain. At 25°C, maximum estimated yields of roquefortine were about 100 mg/liter in the mycelium by 16 days, and no subsequent degradation of this alkaloid was observed. On the other hand, production of PR toxin in the medium peaked at 770 mg/liter at 21 days. At 15°C, yields of roquefortine and PR toxin after 49 days were 60 to 70% of the maximum yields obtained at 25°C. However, about three times more isofumigaclavine A (up to 11 mg/liter) was formed in the mycelium at 15°C than at 25°C. All four strains of *P. roqueforti* produced both roquefortine and PR toxin on the sucrose-yeast extract medium at 25°C; isofumigaclavine A was detected in all but one strain grown on this medium.

Roquefortine is an alkaloid that was recently isolated from *Penicillium roqueforti* (11) and structurally characterized as 10b-(1,1-dimethyl-2-propenyl)-3-(imidazol-4-ylmethylene)-5a,10b,11,11a-tetrahydro-2H-pyrazino[1',2':1,5]pyrrolo[2,3-b]indole-1,4(3H,6H)-dione. Together with isofumigaclavine A, another alkaloid from *P. roqueforti* (11), roquefortine is frequently present in blue cheese (9). Roquefortine is reported to cause convulsive seizures in mice when injected intraperitoneally (11). As large quantities were required for oral toxicity studies, information on whether yields of roquefortine in cultures of *P. roqueforti* could be improved by optimizing the strain, medium, and time of incubation was needed. At the same time, it was hoped to find a way to increase production of isofumigaclavine A, for which yields were extremely low. This compound may be the same as roquefortine A, which was isolated by Ohmomo et al. (6) and reported to show weak pharmacological actions in experimental animals, although the suggested chemical structure differed from that proposed by Scott et al. (11). "PR toxin," a sesquiterpenoid metabolite (15) found to cause liver and kidney damage in the rat, was obtained from *P. roqueforti* cultures in net yields of up to 340 mg of yeast extract-sucrose medium per liter (16). A recent study of PR toxin formation on this medium using the same strain at different temperatures

reported a maximum yield of only 0.72 mg/liter (7). Apart from rice (7), other culture media have not been investigated with regard to the production of PR toxin.

MATERIALS AND METHODS

Organisms. One strain of *P. roqueforti* (strain 596A), originally isolated from Gorgonzola cheese, was obtained from P. Lafont, Unité de toxicologie alimentaire de l'INSERM, 78110 Le Vesinet, France. Strains HPB 061175-1, 061175-3, and 111275 were respectively isolated from samples of Stilton cheese, Danish blue cheese, and Finnish blue cheese selected for their high content of roquefortine (the Stilton contained 3.4 µg/g) or isofumigaclavine A (9). Stock cultures were prepared by growing single, germinated conidia on potato dextrose agar slants for 1 week at 25°C. They were stored at 5°C. Conidia from 5- to 7-day-old subcultures, started from a stock culture and grown at 25°C, served as inoculum for toxin production. Conidia were released from conidiophores by shaking cultures flooded with aqueous 0.05% Tween 80 (polyoxyethylenesorbitan monooleate) on a Vortex mixer. A hemacytometer was used for determining the concentration of conidial suspensions, which were then diluted with sterile water to obtain the desired concentration.

Substrates. The media below were tested for suitability as substrates for roquefortine and PR toxin production. Medium I, a substrate used for the production of a variety of mycotoxins (10), consisted of 15% sucrose and 2% yeast extract (Difco); medium II consisted of a variation of medium I that contained 5% sucrose. Media III and IV were previously used

for alkaloid production by *Aspergillus fumigatus* (12) and *Claviceps paspali* (2), respectively. Medium IV, defined as medium B in reference 2, was supplemented with 0.2 g of L-tryptophan per liter, since L-tryptophan is known to increase alkaloid production by *A. fumigatus* (8), *C. paspali* (1), and *Claviceps purpurea* (13). Medium V consisted of a mineral salts-sucrose solution supplemented with skim milk (14). Media were prepared and sterilized as described in the appropriate references. Two hundred-milliliter portions of medium in 800-ml Roux bottles were inoculated with a small volume (1.2 to 3.0 ml) of a conidial suspension of *P. roqueforti* to give a final concentration of 10^6 conidia per ml of medium. Roux bottles were incubated as stationary cultures in the dark at 25 ± 1 or $15 \pm 1^\circ\text{C}$.

Experiment 1 evaluated metabolite production by the four strains on media I and III at 25°C . Experiment 2 compared the performance of strains HPB 061175-3 and HPB 111275 on media I, II, IV, and V at 25°C . Experiment 3 was a study of the variation of metabolite production by strain HPB 111275 with time at both 25 and 15°C . The three experiments were carried out at different times, but inoculations within each experiment were done on the same day.

Extraction and analysis. After incubation, individual cultures were filtered under reduced pressure for 30 min using Whatman no. 41 filter paper. Mycelia were weighed, wrapped in aluminum foil, and frozen in liquid nitrogen. Both mycelia and media were stored at -25°C until extracted. The pH values of media were estimated with narrow-range indicator strips (EM Reagents). Mycelial mats were blended with 100 ml of acetone for 3 min at high speed in a Waring blender and filtered under reduced pressure (Whatman no. 1 filter paper), and the blender jar and filter mat were rinsed with an additional 40 ml of acetone. Eighty-nine milliliters of water, minus a sufficient amount to allow for an assumed mycelium water content of 80%, was added to the filtrate, which was then extracted twice with 50 ml of chloroform. If necessary, KCl was added to clear emulsions. Media were extracted three times with 100 ml of ethyl acetate; frequently encountered emulsions were separated by centrifugation or by further shaking of the partially separated upper layer. Combined chloroform or ethyl acetate extracts were evaporated under nitrogen on a steam bath and redissolved in 10 or 5 ml of chloroform for initial thin-layer chromatography (TLC).

In suitably diluted mycelium extracts, either before or after separation of the alkaloids by a 0.5 N hydrochloric acid cleanup procedure (9), and in medium extracts after this cleanup step, roquefortine was visually estimated by TLC using the solvent system chloroform-methanol-28% ammonia solution (90:10:1, vol/vol/vol; 9). Isfumumigaclavine A was estimated (9) in the chloroform-diethylamine (4:1, vol/vol) solvent system or, after acid cleanup, in the chloroform-methanol-28% ammonia solution system. Before any hydrochloric acid cleanup procedure, PR toxin was visually estimated on precoated thin layers of Silica Gel DB (Camag) developed with toluene-ethyl acetate-90% formic acid (5:4:1, vol/vol/vol). The standard solution contained 100 μg of PR

toxin per ml of chloroform. Detection was by green fluorescence under longwave ultraviolet light after exposure of the chromatogram to shortwave ultraviolet light for about 0.5 min (16). Roquefortine and isfumumigaclavine A present in low concentrations in some medium extracts could be detected but not reliably estimated in the toluene-ethyl acetate-90% formic acid system. All three metabolites were initially confirmed in extracts of cultures representing different strains of *P. roqueforti* and different media using at least one other TLC solvent system, usually benzene-methanol-acetic acid (24:2:1, vol/vol/vol) for PR toxin.

Isolation and identification of roquefortine, isfumumigaclavine A, and PR toxin. Crystalline roquefortine was isolated from each of the four strains of *P. roqueforti* grown on medium I for 18 or 40 days at 25°C . Extracts of mycelium from two Roux bottles were purified by the acid cleanup procedure and chromatographed on a column of 3 to 5 g of neutral or basic alumina (Woelm, activity grade I). Chromatography was monitored by TLC; roquefortine was eluted by chloroform-ethanol-methanol (98:1:1, vol/vol/vol) and crystallized from methanol-water. Its identity was confirmed by comparison of melting points and infrared and quantitative ultraviolet spectra with those of authentic roquefortine. To obtain crystalline isfumumigaclavine A, together with larger quantities of roquefortine, mycelium from 50 culture bottles of *P. roqueforti* strain HPB 111275 was blended twice with acetone (3 liters, then 1 liter) after 21 to 23 days of growth at 25°C on medium I. The extracts were concentrated separately to 1 and 0.33 liters, water (0.23 liter) was added only to the second extract, and then the extracts were shaken two to three times with chloroform. Cleanup was carried out using 0.5 N HCl essentially as previously referred to (9). Crude alkaloids were chromatographed on 50 g of neutral alumina (Woelm) packed in benzene; column fractions (50 ml) were monitored by TLC. Isfumumigaclavine A was eluted with chloroform (1% ethanol)-benzene (1:1, 3:1, vol/vol) and crystallized from benzene as white plates (5.7 mg) with a melting point of 189 to 190°C and having the same mass and quantitative ultraviolet spectra as standard. Elution of the column with chloroform-ethanol-methanol (97.5:1:1.5-2) yielded roquefortine (662 mg crystallized from methanol-water) with a melting point of 192 to 202°C ($[\alpha]_D^{25} -733^\circ$, $c = 0.1$ in CHCl_3). Spectral identification was made with the addition of the mass spectrum, as done in previous experiments.

PR toxin was isolated from acid-washed extracts of strains HPB 061175-3 (two cultures) and HPB 111275 (one culture) grown for 18 days at 25°C on medium I. Chromatography was carried out on E. Merck Silica Gel 60 (6 and 4.5 g, respectively, for the two extracts), and PR toxin was eluted with chloroform (1% ethanol)-benzene (3:1, vol/vol). Crude PR toxin from strain HPB 061175-3 was further chromatographed (16) on a column prepared with 8 g of Sephadex LH-20, although this procedure appeared to have little effect on purification, and preparative TLC on Silica Gel 7GF (Mallinckrodt) developed with toluene-ethyl acetate-90% formic

acid (5:4:1, vol/vol/vol) was necessary. PR toxin was crystallized three times from benzene-hexane and had a melting point of 152 to 153°C (153 to 153.5°C from strain HPB 111275). Samples were identified by infrared, ultraviolet, and mass spectroscopic comparison with a standard from S. Moreau (5).

RESULTS AND DISCUSSION

Extraction procedures. Acetone was chosen as the solvent for extraction of roquefortine from mycelial mats after a preliminary comparison with the efficiencies of various other solvents, including chloroform, chloroform-28% aqueous ammonia (250:1, vol/vol), chloroform-methanol (3:2, vol/vol), 2% tartaric acid in water-acetone (3:7, vol/vol; 12), and methanol. The amount of roquefortine extracted from the mycelium by an additional blending with 100 ml of acetone was 7.1% (mean of two experiments) of the total extracted. In view of the impracticability of two extractions and the imprecision of visual TLC estimates, this error was considered small enough to be neglected in the results reported in this paper, which were based on a single extraction. The complete extraction of roquefortine by ethyl acetate (three times) from culture media of pH as low as 4.7 was verified by its absence in a fourth extract using ethyl acetate-28% aqueous ammonia (98:2, vol/vol). Virtually complete extraction of PR toxin from culture media was also obtained.

Experiment 1. Comparison of strains. Yields of roquefortine, isofumigaclavine A, and PR toxin by the four strains of *P. roqueforti* under study are shown in Table 1. An incubation period of 18 days was primarily chosen because maximum growth had generally been reached at this time by all isolates.

Medium I was clearly superior to medium III with regard to growth of mycelium and roquefortine production. Most of the roquefortine was present in the mycelium. Medium III supported better production of isofumigaclavine A for strains HPB 061175-3 and HPB 111275, although yields were low. The latter strain appeared to be the best for production of both roquefortine and isofumigaclavine A on medium I and was selected for further study. Since strain HPB 061175-3 was qualitatively different from the other strains because it did not produce isofumigaclavine A on this medium, it was also studied further. All four strains formed PR toxin in medium I by 18 days, but only strains HPB 061175-3 and HPB 111275 produced any, if at all, after 40 days, notably on medium III (up to 5 mg per culture).

Experiment 2. Comparison of media. After the elimination of medium III as a useful substrate for roquefortine production, medium I was compared with three other substrates using strains HPB 061175-3 and HPB 111275 (Table 2). The strains grew best on medium I. Both media I and II were good substrates for roquefortine production at 18 days, but no PR toxin was detected in medium II. Neither strain grew significantly on medium IV, a result that contrasts sharply with the conclusion of Bekmakhanova (3) that a similar medium was suitable for the production of alkaloids in shake culture by several species of *Aspergillus* and *Penicillium*, including *P. roqueforti*. Experiment 2 confirmed that strain HPB 061175-3 did not produce detectable quantities of isofumigaclavine A on sucrose-yeast extract media.

TABLE 1. Comparative production of roquefortine, isofumigaclavine A, and PR toxin by four strains of *P. roqueforti* at 25° C^a

Strain	Medium	Mycelium fresh wt (g)		Roquefortine (mg)				Isofumigaclavine A (mg)				PR toxin (mg) present after 18 days of growth in medium
		18 Days	40 Days	18 Day		40 Day		18 Day		40 Day		
				Mycelium	Medium	Mycelium	Medium	Mycelium	Medium	Mycelium	Medium	
596A	I	31.3	26.8 ^b	14	ND ^c	15 ^b	ND ^b	0.2	ND	0.1 ^b	ND ^b	35
596A	III	17.4	23.9	1	ND	2	ND	ND	ND	ND	ND	0.3
HPB 061175-1	I	29.2	25.4	24	ND	22	ND	0.3	ND	0.3	ND	23
HPB 061175-1	III	15.1	19.2	1	ND	1	ND	0.4	ND	0.3	ND	ND
HPB 061175-3	I	38.5	34.8	18	0.5	17	1	ND	ND	ND	ND	25
HPB 061175-3	III	10.9	15.6	8	0.2	6	0.3	0.5	0.1	0.6	ND	11
HPB 111275	I	25.6	45.2	23	6	59	7	0.4	0.2	0.3	0.2	28
HPB 111275	III	12.2	12.7	1	0.3	4	0.1	2	0.6	2	0.3	18

^a Per Roux bottle containing 200 ml of medium. Mean of duplicate cultures, except where stated.

^b Single determination.

^c ND, Not detectable (<0.1 mg of roquefortine, <0.06 mg of isofumigaclavine A, or <0.05 mg of PR toxin with clean extract).

TABLE 2. Comparative production of roquefortine, isofumigaclavine A, and PR toxin by *P. roqueforti* on four substrates after 18 days at 25°C^a

Strain	Medium	Mycelium fresh wt (g)	Roquefortine (mg)		Isofumigaclavine A (mg)		PR toxin (mg) on medium
			Mycelium	Medium	Mycelium	Medium	
HPB 061175-3	I	35.3	24	0.5	ND ^b	ND	53
	II	22.8	25	2	ND	ND	ND
	IV	0.9	0.4 ^c	2 ^c	ND ^c	ND ^c	ND ^c
	V	17.1 ^c	13	3	ND	ND	3
HPB 111275	I	23.9	23	3	0.5	0.1	4
	II	21.2	28	2	1	0.1	ND
	IV	0.1					
	V	18.5 ^c	3	1	ND	ND	1

^a Per Roux bottle containing 200 ml of medium. Mean of duplicate cultures, except where stated.

^b ND, Not detectable (<0.1 mg of roquefortine, <0.06 mg of isofumigaclavine A, or <0.05 mg of PR toxin with clean extract).

^c Single determination.

Experiment 3. Time-course study of metabolite production. The amount of roquefortine formed in the mycelium by strain 111275 grown on medium I at 25°C essentially paralleled growth (Fig. 1A). Maximum yields of 20 mg of roquefortine per culture (100 mg/liter) occurred by 16 days, and levels remained about the same until the end of the experiment at 49 days. Variability of roquefortine production ascertained from analyses of triplicate cultures was apparent by 28 days and pronounced at 42 days. The yield of 59 mg of roquefortine after 40 days recorded in Table 1 for the same strain on medium I was well above the average found in the time-course study at 42 days. Variability of the fungus was also reflected in variations in final pH (4.4 to 7.2 at 28 days and 4.6 to 7.6 at 42 days) and in appearance of the mycelial mats, indicating autolysis. The amount of roquefortine excreted into the medium did not exceed a mean of 4 mg per culture. The yields of isofumigaclavine A determined from 7 to 35 days were consistently low (0.5 to 0.7 mg per culture in the mycelium and up to 0.2 mg in the medium). Yields of PR toxin in the medium rose steeply to a maximum at 21 days incubation; much smaller amounts were found in the mycelium (Fig. 1). By comparison, amounts of PR toxin found in the medium in experiments 1 and 2 after 18 days (Tables 1 and 2) were much lower than expected from Fig. 1. This suggests that the exact time of the rapid increase of production of PR toxin is sensitive to unknown factors, and any experiment designed to obtain PR toxin should include a daily sampling and analysis to check that production is nearing the maximum.

P. roqueforti strain HPB 111275 showed good growth at 15°C by 35 days (Fig. 1B). Cultures

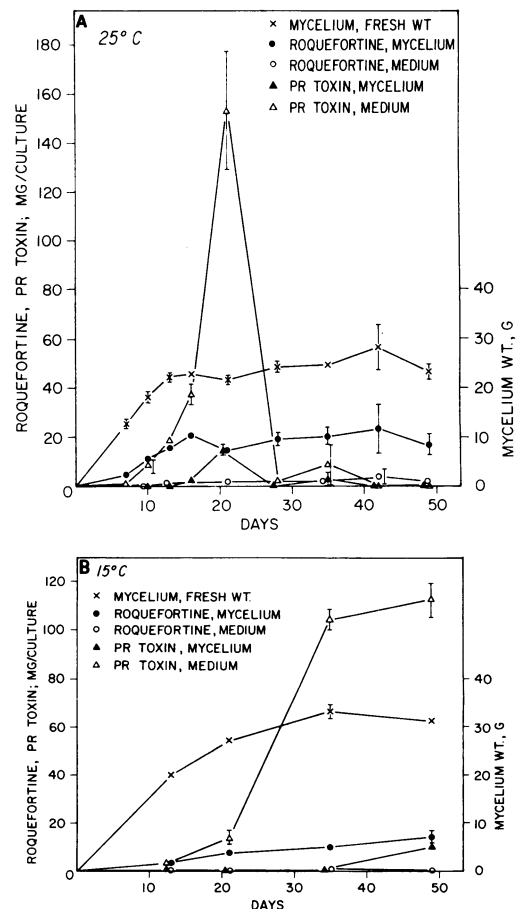


FIG. 1. Production of roquefortine and PR toxin by *P. roqueforti* strain HPB 111275 on 200 ml of 15% sucrose-2% yeast extract (medium I) at (A) 25°C and (B) 15°C. Vertical bars indicate standard error (if greater than size of point symbol) in triplicate cultures.

showed less variation than at 25°C. Yields of roquefortine and PR toxin at 49 days were 60 to 70% of maximum yields obtained at 25°C. However, the mean amounts of isofumigaclavine A, already formed by 13 days and still present at 49 days, were 2 mg per mycelial mat, about three times that formed at 25°C; none was detected in the medium. These yields of isofumigaclavine A obtained at 15°C on medium I are comparable to those obtained on medium III at 25°C (Table 1). Further work is still required to find conditions suitable for production of this clavine alkaloid in quantities sufficient for toxicological investigation. The fact that isofumigaclavine A yields exceeded those of roquefortine in several commercial blue cheese samples (9), including that from which strain HPB 111275 was isolated, should encourage such research. It is of interest that blue cheese itself is generally ripened by storage at 9 to 12°C for 3 months (4).

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