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STUDIES ON THE PR TOXIN OF PENICILLIUM ROQUEFORTI

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Abstract

A mycotoxin, confirmed by chemical, physical and spectroscopic data as the PR toxin described by Ru-Dong Wei and coll. (15) has been isolated from culture filtrates of *Penicillium roqueforti* Thom. Factors affecting the toxin and mycelium production, acute and chronic toxicity in experimental animals and the frequency of toxinogenesis of 2I isolates of *P. roqueforti* (including a brown mutant) isolated from different materials, foods especially, were also studied. An hypothesis on the absence of PR toxin in cheeses fermented with *P. roqueforti* is also advanced.

Introduction

Toxic metabolites from *Penicillium roqueforti* Thom have been reported by several authors (2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14). Ru-Dong Wei and coll. (15) isolated and identified a mycotoxin from culture filtrates, named as PR toxin. In view of a study of toxinogenic fungi in food products, a research program on the significance of this mycotoxin with relation to fermented cheeses was started. This paper deals with the results of our investigations on the occurrence and toxicity of PR toxin.

Materials and Methods

Cultures

P. roqueforti NRRL 849 type culture was used for isolation and characterization of PR toxin. Twenty other isolates of *P. roqueforti* in our collection, PR I, PR 2, PR 3, PR 4, PR 5, PR 6, PR 7, PR 8, PR 9, PR 10, PR 11, PR 12, PR 13, PR 14, PR 15, PR 10B, PR 60B, PR 93B, PR 94, PR 95 and a brown mutant of *P. roqueforti*, CBS 50175, were used for the study of the frequency of toxinogenesis.

Production of PR toxin

For toxin production a standard medium containing 2% yeast extract and 15% sucrose was used (pH 5.5). Half liter flasks containing 100 ml of medium per flask were sterilized at 121 °C for 20 minutes. The flasks were inoculated with a suspension of conidia (10^6–10^8) of *P. roqueforti* and incubated for 14 days at 24 °C in a stationary condition in darkness. Production of the toxin in light, at different temperatures, in dark and light shaken conditions, with different pH values of the initial medium, with medium containing different concentrations of sucrose, in aerobic, microaerophilic (CO_2 seal) and anaerobic (Gas Pak) conditions were comparatively studied. Toxin production in parallel with mycelium production (dry weight) and pH variations of the medium were studied from the first to the 210th day of incubation. Starting on the 14th day, 30 ml of fresh medium were added each week to the flasks. The culture liquid, obtained by filtration and squeezing the mycelium, was mixed with five volumes of chloroform. The extracted fractions were treated with Na_2SO_4 to remove any traces of water, then filtered again to remove Na_2SO_4 and evaporated in a rotary evaporator to release the chloroform. In this manner a crude oily extract was finally obtained.

Purification by chromatography

The crude extract from the culture filtrates was dissolved in a small amount of benzol – acetate (9 : I) and applied to a 3 by 70 cm silica column. The column was placed at 5 °C and eluted with the same solvent. Ten ml fractions were collected and examined by thin layer chromatography

(same eluent and U.V. rays and H_2SO_4 as developers) in comparison with PR toxin (kindly furnished from Dr. Ru-Dong Wei). Fractions containing PR toxin were combined, evaporated and concentrated in vacuo at 30–40 °C to a glassy solid that spontaneously crystallized.

Identification

Identification of the PR toxin was carried out with the following tests:
a) TLC of a mixture with the pure specimen of PR toxin and its behavior under U.V. light (blue fluorescence) and cold and hot H_2SO_4 (yellow and brown color).
b) U.V. spectrum. (EtOH): λ_{max} 248 nm (ε = 9000).
c) I.R. spectrum. ($CHCl_3$): 2945–1735–1720–1620–1460–1435–1380–1245 and 1035 cm^{-1}.
d) MNR spectrum. (60 MHz, $CDCl_3$, ϑ): 9.6 (IH, s, CHO); 6.4 (IH, s, insaturated proton); 1.15 (IH, X part of ABX); 3.9 (IH, dd); 3.65 (IH, d, AB part of ABX); 2.15 (3H, s, $-O-CO-CH_3$); 1.48 (3H, s, tertiary CH_3); 1.45 (3H, d, J: 0.8 Hz, CH_3); 1.04 (3H, d, J = 7 Hz, secondary CH_3); 2.3–1.65 (2H, dd AB system, J = 14 Hz, $-CH_2-$); 1.7–1.3 (IH, m).

Biological assay

Toxicity was studied by intraperitoneal inoculation of proportional amounts of the toxin in solution in 0.2 ml of propylene glycol in weanling male and female rats (50–60 gr). Rats of the same body weight were inoculated intraperitoneally with an equal amount of propylene glycol only, as a control. Chronic toxicity was subsequently studied by oral administration of the toxin dissolved in the drinking water in 12 weanling male and female rats (60 gr). Additional pathogenicity studies were conducted by inoculating a conidial suspension (10^6–10^8) in the tail veins of four mice (25 gr).

Cheese assay

Various samples of commercial cheese fermented with *P. roqueforti* were also tested to detect any eventual presence of PR toxin. An emulsion of the cheese was filtered and mixed with five volumes of chloroform. The mouldy fractions removed from the cheese were also tested.

Results

Biological properties

The PR toxin was found in every culture of *P. roqueforti* tested. Within 10–15 minutes after 1.5 mgr of PR toxin was injected intraperitoneally, all of the inoculated rats developed breathing difficulties, motor incoordination, flaccid paralysis, especially in the back legs, inability to support their body weight. Death occurred within 2–6 hours. Histological tests showed a turbid swelling of the hepatocite cytoplasm in the earlier dead animals. Clearer lesions, with vacuolar degeneration, were found in the hepatocites of the rats inoculated with the lower amounts of PR toxin and that had died after two days (6). The DL_{50}, according to the formula of Reed and Muench, was 14.5 mgr/Kg body weight. Control animals, intraperitoneally inoculated witk 0.2 ml of propylene glycol (obmitting the toxin), did not show any effect. Rats orally administered during two months with 0.5 mgr of toxin pro capite/pro die (on an average) did not show any visible effect eleven months after suspension of treatment. The observation period will be extended to determine if there is an eventual cancerogenic role of PR toxin, comparing with the feeding test of Frank and coll. (I) over rats with commercial starters for blue cheese and commercial cheese. Inoculation of spore suspensions in the tail veins of mice revealed the saprophytic survival of *P. roqueforti* up to five – six weeks in the organs of the sacrificed animals.

Structure of PR toxin

Chemical, physical and spectroscopic data led us to identify the isolated mycotoxin as the PR toxin, whose structure had been established by Ru-Dong Wei and coll. (15).

Conditions for PR toxin production

In our preliminary experiments toxin production was obtained by incubating the cultures of *P. roqueforti* for 14 days at 24 °C in darkness. PR toxin production in darkness and in light was comparatively studied under different periods of incubation (Fig. I). The production of the toxin started during the ninth day either in darkness or in light. The amount was essentially equal under both conditions. The PR toxin was not produced either in darkness or in light in shaken cultures. After the ninth day the amount of the toxin gradually increased up to the 35th day. It slowly

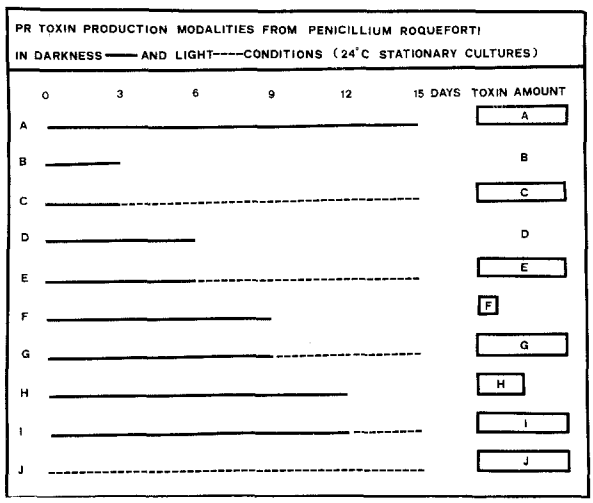

Fig. 1.

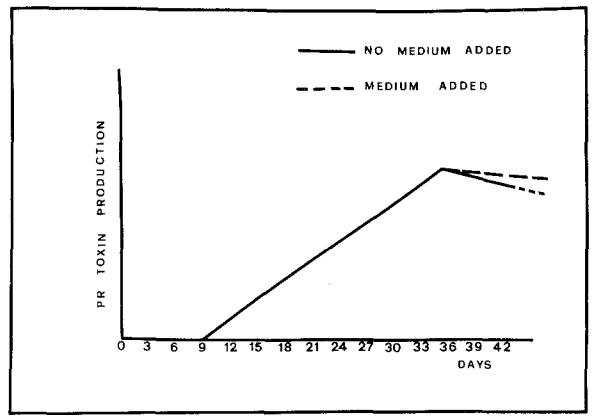

Fig. 2. Catabolism modalities of PR toxin.

decreased afterwards disappearing by the 120th day. The weekly addition of fresh medium seemed to reduce the decrease of toxin (Fig. 2). During the 35th day of incubation, pH variations and mycelium weight occurred. The amount of toxin decreased with the alkalinization of the medium (Fig. 3). PR toxin production occurred within pH 4.5–9 with its optimum at pH 5.5 in a 14 day incubation period. The greatest mycelium weight was achieved at pH 5.5. Mycelium was formed slowly or in small amounts when the pH moved toward acidity or alkalinity (Fig. 4). Daily observation of pH variations emphasized pH moved with the 14th day toward the optimum for the production of the toxin in the cultures with pH initial values allowing toxin production (pH 4.5–9). This condition did not occur, on the contrary, when the initial pH of the medium was either too acid or basic (Fig. 5). The production of PR toxin and mycelium was also influenced by the temperature of incubation. The toxinogenesis occurred within the temperature range of 10–30 °C with the optimum temperature at 24 °C (Fig. 6). Mycelium only with no PR toxin developed at 4 °C. Mycelium and PR toxin were not

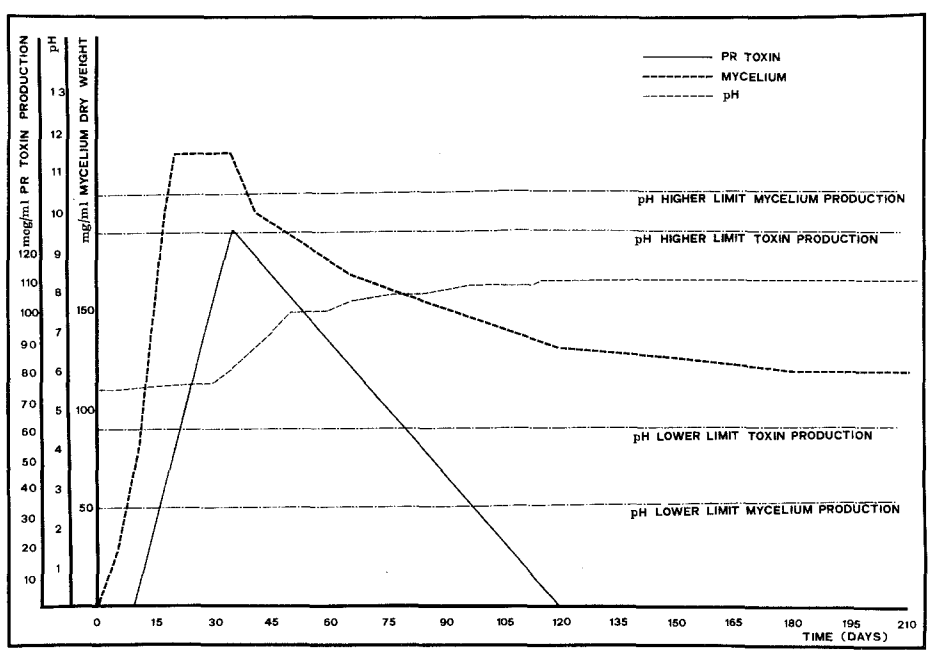

Fig. 3. pH, Mycelial weight and PR toxin production variations in the time.

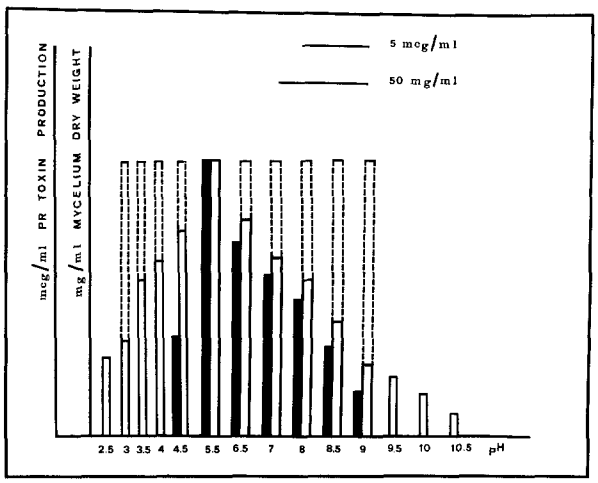

Fig. 4. pH Influence on mycelial weight and PR toxin production.

■: PR toxin amount; □: mycelium dry weight; ⬚: delayed increase of mycelium dry weight (14 day incubation period).

produced at 37 °C. The percentage of sucrose in the medium influenced the production of mycelium and toxin, which started to appear at 1 % and 5 % sucrose, to a maximum at 25 % and 15 % respectively. The amount of mycelium and toxin was practically stable up to a concentration of 35 % sucrose (Fig. 7). Mycelium but not PR toxin was formed under the microaerophilic conditions (Fig. 8).

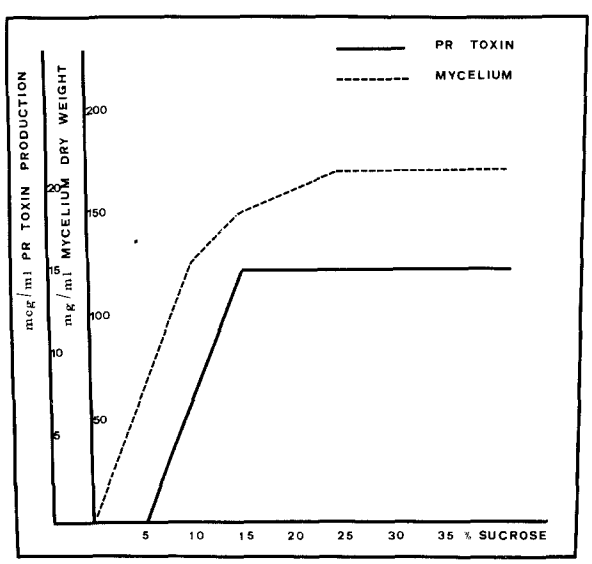

Fig. 7. Influence of sucrose concentration on mycelial weight and PR toxin production.

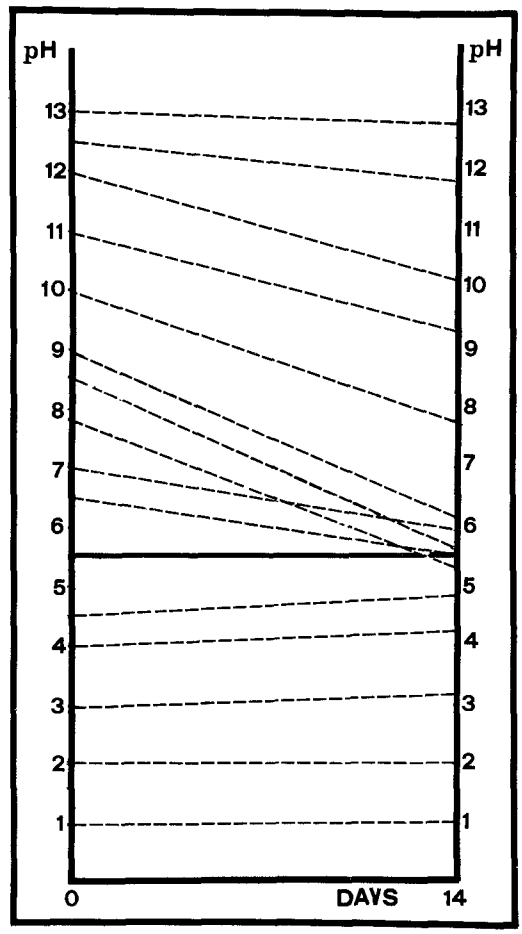

Fig. 5. Modification of various initial medium pH values during 14 days of incubation.

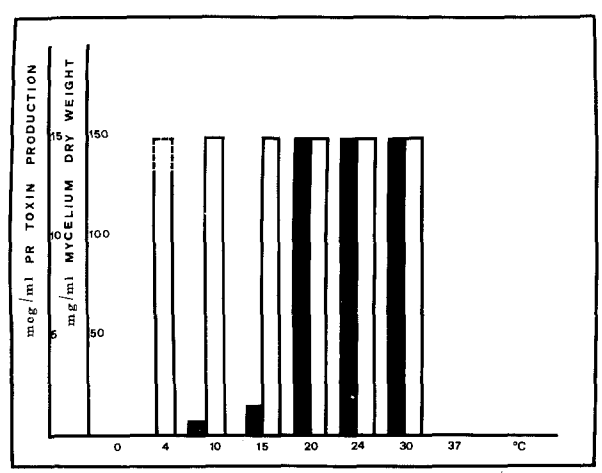

Fig. 6. Temperature influence on mycelium weight and PR toxin production.

■: PR toxin amount; □: mycelium dry weight; ⬚:delayed increase of mycelium dry weight (14 day incubation period).

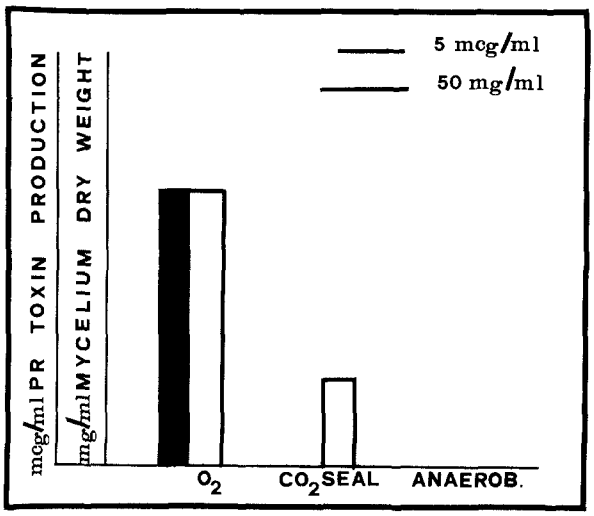

Fig. 8. Influence of O_2 concentration on mycelial weight and PR toxin production.

■: PR toxin production; □: Mycelium dry weight (14 day incubation period).

FREQUENCY OF P. ROQUEFORTI STRAINS PRODUCING PR TOXIN

NO	STRAIN	ISOLATED FROM:	PR TOXIN
1	PR NRRL 849	CHEESE	+
2	PR 10 B	CHEESE	+
3	PR 60 B	GORCONZOLA	+
4	PR 93 B	SMOKED CHEESE	+
5	PR 94	BLUE CHEESE	+
6	PR 95	GORGONZOLA	+
7	PR 1	STRAWBERRY JAM	+/–
8	PR 2	MEAT	+
9	PR 3	MEAT	+
10	PR 4	SOIL	+
11	PR 5	MEAT	+
12	PR 6	GERMAN CHEESE	+
13	PR 7	DANISH CHEESE	+
14	PR 8	CHEESE	+
15	PR 9	CHEESE	+
16	PR 10	CHEESE	+
17	PR 11	CHEESE	+
18	PR 12	GERMAN CHEESE	+
19	PR 13	GORGONZOLA	+
20	PR 14	EGG-PLANTS IN OIL	+
21	PR 15	CHEESE	+
22	PR CBS 50175	BARLEY	+

Fig. 9.

Frequency of isolates producing PR toxin

In a further study the frequency of toxinogenesis of 21 cultures of *P. roqueforti*, including a brown mutant, was investigated. These cultures had been isolated from different substrates. Each tested culture of *P. roqueforti*, including the brown mutant, produced PR toxin. The PR I isolate showed low toxic activity (Fig. 9).

Cheese assay

The performed test on the whole cheese and the mouldy fractions only did not yield any indication for the presence of PR toxin.

Discussion

Our results confirmed that the PR toxin is produced only in stationary cultures, either in darkness or in light, within temperature, pH, sucrose and O_2 concentrations limits narrower than the limits required for the production of mycelium. PR toxin production starts at the ninth day of incubation, increases progressively up to the 35th day and successively decreases and finally disappears at the 120th day approximately. The observation that each tested isolate of *P. roqueforti* produced PR toxin does not contradict, in our opinion, our repeated previous studies that PR toxin is not produced in cheese (Gorgonzola, Roquefort, Blue Cheese). This discordance can be attributed to the conditions that prevail during cheese production (microaerophilic environment and low carbohydrates), stagioning (acid environment) and maintenance (temperature) of the cheese, factors that experimentally have been shown to favor the growth of mycelium but not the production of PR toxin.

Acknowledgement

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References

1. Frank, H.K., R. Orth, S. Ivankovic, M. Kuhlmann & D. Schmahl. 1977. Investigations on carcinogenic effects of Penicillium caseicola and P. roqueforti in rats. Experientia 33 (4) 515–516.
2. Kanota, K. 1970. Studies on toxic metabolites of Penicillium roqueforti. Proc. Ist U.S. – Japan Conf. Toxic Microorganisms, U.S. Dept. Interior unnumbered publications, Washington D.C., 129–132.
3. Lafont, P., J. Lafont, J. Payen, E. Chany, G. Bertin & C. Frayssinet. 1976. Toxin production by 50 strains of Penicillium used in the cheese industry. Food and Cosmetics Toxicology 14 (2) 137–139.
4. Moreau, S., A. Gaudemer, A. Lablache-Combier & J. Biguet. 1976. Metabolites de Penicillium roqueforti: PR toxine et metabolites associes. Tetrahedron Lett. 833.
5. Moreau, S., M. Cacan & A. Lablache-Combier. 1977. Eremofortin C. A new metabolite obtained from Penicillium roqueforti cultures and from biotransformation of PR toxin. J. Org. Chem. 42 (15) 2632–2634.
6. Moule', Y., M. Jemmali & M. Rosseau. 1976. Mechanism of the inhibition of transcription by PR toxin, a mycotoxin from Penicillium roqueforti. Chem. Biol. Interactions 14 (3 and 4) 207–216.
7. Ohmomo, S., T. Sato, T. Utagawa & M. Abe. 1975. Isolation of festuclavine and three new indole alkaloids, roquefortine A, B and C from the cultures of Penicillium roqueforti. Agr. Biol. Chem. 39 (6) 1333–1334.
8. Orth, R. 1976. PR Toxinbildung bei Penicillium roqueforti-Stammen. Z. Lebensm. Unters. Forsch. 160, 131–136.
9. Piva, M., T. Guiraud, J. Crouze & P. Galzy. 1976. Effect of cultures conditions on mycotoxin excretion by some strains of Penicillium roqueforti. Le Lait 56 (557) 397–406.
10. Scott, P.M., M.A. Merrien & J. Polonsky. 1976. Roquefortine and isofumigaclavine A, metabolites from Penicillium roqueforti. Experientia 32 140.
11. Scott, P.M. & B.P.C. Kennedy. 1976. Analysis of Blue Cheese for roquefortine and other alkaloids from Penicillium roqueforti. J. Agric. Food Chem. 24 (4) 865–868.
12. Scott, P.M., B.P.C. Kennedy, J. Harwig & B.J. Blanchfield. 1977. Study of conditions for production of roquefortine and other metabolites of Penicillium roqueforti. Appl. Environ. Microbiol. 33 (2) 249–253.
13. Still, P.E., R.D. Wei, E.B. Smalley & F.M. Strong. 1972. A mycotoxin from Penicillium roqueforti isolated from toxic cattle feed. Fedn. Proc. Fedn. Am. Socs. exp. Biol. 31, 733.
14. Wei, R.D., P.E. Still, E.B. Smalley, K.H. Schnoes & F.M. Strong. 1973. Isolation and partial characterization of a mycotoxin from Penicillium ruqoeforti. Appl. Microbiol. 25 (I) 111–114.
15. Wei, R.D., K.H. Schnoes, P.A. Hart & F.M. Strong. 1975. The structure of PR toxin, a mycotoxin from Penicillium roqueforti. Tetrahedron 31, 109–114.