Studies on the PR toxin of *Penicillium roqueforti*

Shenq-Chyi Chang,1 Yau-Huei Wei,1 Kuang-Lieh Lu,2 Min-Kuei Cheng,1 Ding-Ling Wei,3 Shung-Chang Jong,4 and Ru-Dong Wei1

1. Department of Biochemistry, National Yang-Ming University, Taipei 112, Taiwan, Republic of China.
2. Institute of Chemistry, Academia Sinica, Taipei 112, Taiwan, Republic of China.
3. Division of Biology, Center for General Education, National Yang-Ming University, Taipei 112, Taiwan, Republic of China.
4. Mycology Department, American Type Culture Collection, Manassas, Virginia 20110, U.S.A.

ABSTRACT

*Penicillium roqueforti* Thom is a fungus traditionally used in the ripening of French Roquefort cheese. PR toxin (C\textsubscript{17}H\textsubscript{20}O\textsubscript{6}) is a secondary metabolite of the fungus *P. roqueforti*. The toxin has been shown to be lethal to animals and exhibits a broad spectrum of biochemical activities which cause toxicities in animals. In the past few years, we have come across several secondary metabolites related to PR toxin and observed that they are produced sequentially in the culture medium of the fungus. The compounds were isolated, purified and identified as eremofortin C (EC) (C\textsubscript{17}H\textsubscript{22}O\textsubscript{6}), PR-imine (C\textsubscript{17}H\textsubscript{21}O\textsubscript{5}N), PR-acid (C\textsubscript{17}H\textsubscript{20}O\textsubscript{7}), and PR-amide (C\textsubscript{17}H\textsubscript{21}O\textsubscript{6}N), respectively. The structures of EC, PR toxin, PR-acid and PR-amide are closely related and differ only in the presence of a hydroxymethyl group in EC, an aldehyde group in PR toxin, a carboxyl group in PR-acid and an amide group in PR-amide at the C-12 position, respectively. As our effort to elucidate the synthetic and metabolic pathway of the toxin, we discovered that EC is transformed to PR toxin by EC oxidase and PR toxin is further converted into PR-acid by PR oxidase. Moreover, the PR-acid was metabolized into PR-amide by PR-amide synthetase. Thus, we propose that PR toxin is synthesized from EC and is degraded into PR-acid and PR-amide in the culture medium of *P. roqueforti*.

Key words: Eremofortin C, *Penicillium roqueforti*, PR-acid, PR-amide, PR-imine, PR toxin.

Introduction

*Penicillium Roqueforti* is a fungal species traditionally used in the ripening of French Roquefrot cheese. Since the discovery of PR toxin by Wei et al. (1973), several other secondary metabolites related to PR toxin by *P. roqueforti*, such as eremofortins A, B, C, D, and E, have been isolated and characterized (Cacan et al., 1977; Moreau et al., 1977, 1980a, b). Among these metabolites, only the PR toxin is lethal to rats, mice, and cats (Chen et al., 1982; Wei et al., 1975). PR toxin inhibits RNA and protein synthesis (Moule et al., 1976; 1978); the activities of the DNA polymerases \(\alpha, \beta,\) and \(\gamma\) (Lee Wu et al., 1984; Wei et al., 1985); and mitochondrial respiration and oxidative phosphorylation in animal cells (Wei et al.,...
1985, Wei et al., 1984). It also alters the genetic activities of *Saccharomyces cerevisiae* and *Neurospora crassa* (Wei et al., 1979).

Since PR toxin was the only toxic secondary metabolite that has been isolated from the culture medium of *P. roqueforti* (Wei, 1973), it has been our interest to elucidate its mechanism of biosynthetic and metabolic pathway.

**Materials and methods**

**Organisms and growth condition**

*P. roqueforti* ATCC 48936 were maintained on potato dextrose agar slants at 4°C.

To enhance high levels of PR toxin and other secondary metabolites, we grew the fungus in YESC medium containing 1% yeast extract, 7.5% sucrose, and 20% corn extract (Chang et al., 1991a, 1991b). A Roux bottle (600 mL) containing 150 mL of liquid medium was inoculated to a final concentration of $5 \times 10^4$ conidia per mL and incubated at 24°C as stationary culture.

**Extraction and purification of eremofortin C (EC), PR toxin, PR-imine, PR-acid, and PR-amide**

We monitored the production of EC, PR toxin, PR-imine, PR-acid, and PR-amide in the culture medium throughout the growth. The culture medium was acidified with hydrochloric acid to pH 3 and then extracted with an equal volume of chloroform. The chloroform extracts were collected and concentrated in a rotary evaporator to the density of a thick brown syrup. Further purifications of EC, PR toxin, PR-imine, PR-acid, and PR-amide were performed according to previously published methods (Chang et al., 1991a, 1991b, 1993, 1996). The pure compounds were then dried under N$_2$ gas and stored at –20°C.

**Quantitative analysis of EC, PR toxin, PR-imine, PR-acid, and PR-amide**

The five compounds were quantitatively analyzed by high performance liquid chromatography (HPLC, Waters, Milford, U.S.A.). A Water model 510 liquid chromatograph equipped with a Water 486 tunable detector set at 254 nm was used with a Water 740 data module. Separation was achieved with a Cosmosil silica column (25 cm by 4.6 mm) for EC, PR toxin, PR-imine, and PR-amide, and a Cosmosil 10 C$_{18}$ column (25 cm by 4.6 mm) for PR-acid. EC and PR toxin were measured with chloroform as the solvent at a flow rate of 2 mL/min. PR-amide and PR-imine were assayed with solvents of 1 and 3% methanol in chloroform, respectively, at a flow rate of 2 mL/min. PR-acid was assayed with methanol-H$_2$O (2:3, v/v) containing 20 mM citrate-phosphate buffer (pH 3) at a flow rate of 1.5 mL/min.

**Characterization of PR-imine, PR-acid, and PR-amide**

The chemical structures of these compounds were determined by elemental analysis and spectroscopic measurements. Elemental analysis data were obtained with a Perkin-Elmer model 240C elemental analyzer. Electron impact mass spectra were obtained with a VG 70-250S mass spectrometer at 20 eV for PR-imine and 50 eV for PR-amide and with a JEOL SX-102A mass spectrometer at 70 eV for PR-acid. UV spectra were recorded on a Perkin-Elmer Lambda-5 spectrophotometer. Infrared spectra
were determined with a Perkin-Elmer model 983G infrared spectrophotometer. Both $^1$H and $^{13}$C nuclear magnetic resonance NMR spectra were recorded on a Bruker AM-400 NMR spectrometer.

**Crystals of PR-amide**

The compound PR-amide was isolated and purified. Crystals of the colorless PR-amide were grown by diffusing n-hexane into a saturated CH$_2$Cl$_2$/CH$_3$OH solution of the compound at room temperature. Structure solved by direct method using MULTAN and the remaining atoms were located in succeeding difference Fourier synthesis. H atoms found in difference Fourier map after isotropic refinement and them refined. Computing programs were performed with NRCC package on microvax 3600 computer (Gabe *et al*., 1985).

**Purification of the enzymes**

When the yields of EC oxidase, PR oxidase and PR-amide synthetase reached their maxima, the culture medium or mycelium was harvested accordingly. Further purifications of these enzymes were performed according to previous methods (Chang *et al*., 1985, 1998, 2004).

**Assay for EC oxidase, PR oxidase and PR-amide synthetase activity**

The activity of EC oxidase, PR oxidase, and PR-amide synthetase were determined by HPLC analysis (Chang *et al*., 1985, 1998, 2004). One unit of the enzyme activity was defined as the amount of enzyme that catalyzed the transformation 1 µmol of EC to PR toxin per min for EC oxidase and 1 µmol of PR toxin to PR-acid per min for PR oxidase and 1 µmol of PR-acid to PR-amide per min for PR-amide synthetase under the assay conditions.

**Results**

The EC, PR toxin, PR-imine, and PR-amide were detected by HPLC with normal phase system, while the high polarity of PR-acid was measured in a reverse phase system. These compounds could be availably separated by HPLC methods (Fig. 1).

In the time-course study we found that the yields of EC, PR toxin, PR-imine, PR-acid, and PR-amide by *P. roqueforti* ATCC 48936 peaked on the 13th, 14th, 17th, 21th, 23th, days in the culture medium at 24°C, respectively (Fig. 2 and 3). Moreover, we found that the PR-imine in the culture medium was unstable, decreasing from the maximum to zero within 2 days. On the other hand, the yields of PR-acid and PR-amide were stable over a long period in the culture medium (Fig. 2 and 3). The dimension of PR-amide crystal was 0.36 × 0.26 × 0.20 mm. The molecular structure of the compound was shown in Fig. 4. The crystal structure of PR-amide compares closely with the crystal structure of PR toxin (Wei *et al*., 1975) with an amide instead of the aldehyde group.

The peak amount of EC occurs earlier than that of PR toxin, and the decrease in the amount of EC is always associated with a rapid increase in PR toxin production. We found that EC is the direct precursor of PR toxin and that EC oxidase is responsible for such a transformation. In addition, the enzyme was found to be a glycoprotein containing a single polypeptide, the sugar residues constitute 6.5% of the
Fig. 1. HPLC chromatograms of PR toxin and PR-imine standards (a), PR-amide standard (b), PR-acid standard (c). The analysis proceeded as described in the text.

Fig. 2. Thin layer chromatogram of chloroform : acetic acid (6:1) extracts from the culture medium of *P. roqueforti*. The silica gel plate was developed with *n*-hexane : chloroform : acetic acid (2:6:1). The spots were visualized by charring with sulfuric acid and heating at 110°C for 10 min. The numbers indicate the day of harvest.
The molecular weight of the EC oxidase was estimated by SDS-PAGE to be ca. 62 kDa (data not shown).

Moreover, we observed that PR toxin appeared earlier than PR-acid and that with time the amount of PR toxin decreased while that of PR-acid concomitantly increased. When PR toxin was incubated in the buffer solution containing the culture medium, PR-acid appeared and the amount of PR toxin decrease. Since the enzyme catalyzed the oxidation of the –CHO group of PR toxin to the –COOH group of PR-acid, the enzyme must be either an aldehyde dehydrogenase or an oxidase. We found that the enzyme does not require NAD$^+$ or NADP$^+$ as a cofactor to catalyze the transformation. By using the coupling assay in the presence of peroxidase and 4-aminoantipyrine plus phenol, we
were able to determine the oxidase activity of the enzyme. SDS-PAGE revealed that the molecular mass of the PR oxidase was approximately 88 kDa (data not shown). On the other hand, we observed that PR-imine was also detected when PR toxin was degraded in the culture medium (Fig. 2 and 3). Since gas chromatography-mass spectrometry analysis indicated the presence of NH$_3$ in the culture medium, it is conceivable that PR-imine is one of degradative metabolites of PR toxin in the culture medium.

The production of PR-amide was also related to the disappearance of PR toxin. The time-course study showed that the peak amount of PR-acid and PR-amide appeared on the 21th and 23th days in the culture medium, respectively (Fig. 2 and 3). They differ by only a carboxyl group (PR-acid) and an amide (PR-amide) at the C-12 position. We discovered that PR-amide synthetase in the mycelium of the fungus was responsible for the transformation of PR-acid into PR-amide. The enzyme has a molecular mass of approximately 66 kDa, and it is a monomer (data not shown).

**Discussion**

Since PR toxin was the first and only toxic secondary metabolite that has been isolated from the culture medium of *P. roquefrti*, it has been our interest to elucidate its mechanism of biosynthetic and degradative pathway. As part of our effort to understand the biosynthetic pathway of the toxin, we discovered the EC oxidase that catalyzes the conversion of EC to PR toxin. The time-course study showed that the peak amount of PR toxin occurred earlier than those of PR-imine, PR-acid, and PR-amide. The decrease in the level of PR toxin was always associated with an increase in the other three compounds (Fig. 2 and 3). The chemical structures of PR toxin and these compounds are closely related (Chang et al., 1993, 1996; Wei et al., 1975). PR toxin appears to be the precursor of PR-imine, PR-acid, and PR-amide.

Moreover, we discovered that PR oxidase in the culture medium of the fungus was responsible for the transformation of PR toxin into PR-acid and PR-amide synthetase in the culture mycelium was responsible for the transformation of PR-acid into PR-amide. On the other hand, ammonia was produced in the culture medium of the fungus when it was grown more than 15 days old. It is conceivable that PR toxin reacted with NH$_3$ in the culture medium to produce PR-imine. Therefore, we propose a hypothetical biosynthetic and metabolic pathway for PR toxin of *P. roqueforti* (Fig. 5).

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**References**

Biosynthetic and metabolic pathway of PR toxin

Fig. 5. A proposed biosynthetic and metabolic pathway for PR toxin of *P. roqueforti.*


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