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Paecilotoxin production in clinical or terrestrial isolates of *Paecilomyces lilacinus* strains

Yuzuru Mikami[1], Katsukiyo Yazawa[1], Kazutaka Fukushima[1], Tadashi Arai[1], Shun-ichi Udagawa[2] & Robert A. Samson[3]

[1] *Department of Experimental Chemotherapy, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1, Inohana, Chiba, Japan 280;* [2] *Division of Microbiology, National Institute of Hygienic Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo, Japan 158;* [3] *Centraalbureau voor Schimmelcultures, 3740 AG Baarn, The Netherlands*

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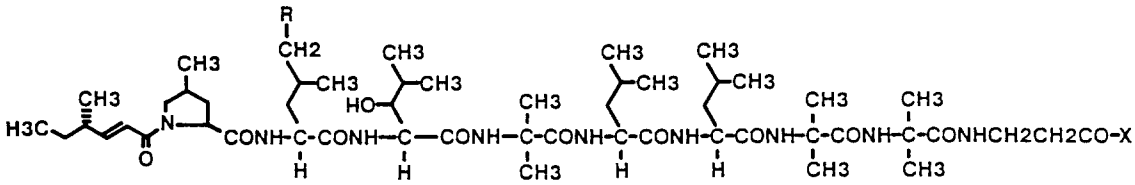
Abstract

The production of paecilotoxin from various isolates of *Paecilomyces lilacinus* was studied using three different media and high performance liquid chromatography (HPLC). Alkaline medium was found to be suitable for the production of the toxins. Among 20 strains tested, 19 including four clinical isolates were found to produce the toxins. Production patterns of paecilotoxins were very similar in each strain and the main toxins were A and B.

Introduction

Paecilomyces lilacinus (Thom) Samson is a common hyphomycetes, which occurs in the soil, on insects or nematodes. However, an increased number of clinical cases due to this fungus have been recognized [1, 2]. We have reported on toxic substances, called paecilotoxins (leucinostatins) which are produced by *P. lilacinus* [3, 4]. Paecilotoxins are neutral straight peptide toxins, which include unsaturated fatty acid in the N-terminal group and amine in the C-terminal. Paecilotoxins are also characterized as having a high content of a peculiar amino acid α-aminoisobutyric acid (Fig. 1). Biological studies of the toxins showed that they have strong uncoupling activity against rat liver mitochondria as well as antimicrobial activity [4]. Our previous studies demonstrated that paecilotoxins belong to the group of mycotoxins with the highest oral toxicity reported to date [4]. We also confirmed their active role as etiological agents in a fungal infection model of rabbit using *P. lilacinus* conidia. Since *P. lilacinus* has been frequently isolated from various habitats, we were interested in whether the strains from these different sources actually produce paecilotoxins. This paper reports on the studies on paecilotoxin production by *P. lilacinus* strains collected from various sources including clinical isolates.



Paecilotoxin (Leucinostatin)	R	X
A	CH(OH)CH2COCH2CH3	NHCH(CH3)CH2N(CH3)2
B	CH(OH)CH2COCH2CH3	NHCH(CH3)CH2NHCH3
C	H	NHCH(CH3)CH2N(CH3)2
D	H	NHCH(CH3)CH2NHCH3
H	H	NHCH(CH3)CH2NO(CH3)2
K	CH(OH)CH2COCH2CH3	NHCH(CH3)CH2NO(CH3)2

Fig. 1. Structure of paecilotoxins A, B, C, D, H and K.

Materials and methods

Organisms

Twenty strains of *P. lilacinus* were collected and maintained by transferring to potato-dextrose agar (PDA) slant (Difco) or the freeze-drying method. The origins of all strains are shown in Table 1. The spores from recovered cultures from the freezed-dried materials or the slant cultures were inoculated onto PDA slant and incubated for 7 days at 27 °C.

Production of paecilotoxins

The following three media were used: Medium A (g/l): sucrose, 50.0; corn steep liquor, 10.0; K_2HPO_4 10.0; $MgSO_4 \cdot 7H_2O$ 2.0; tap water to 1000 ml. The pH of the medium was adjusted between 4.2 and 4.8 before autoclaving. Medium B: sucrose, 50.0; peptone (Nissui, Japan), 5.0; yeast extract (Difco), 5; K_2HPO_4, 1.0; Na_2CO_3, 10.0; $MgSO_4 \cdot 7H_2O$, 0.2; tap water to 1000 ml. The pH was adjusted to 8.2 to 9.0 before autoclaving. Medium C: sucrose, 50.0; peptone, 5.0; yeast extract, 5.0; K_2HPO_4, 0.2; $CaCO_3$, 5.0; tap water to 1000 ml. The pH was adjusted to 6.5 to 7.2. The mycelial fragments from PDA slants were inoculated into a 500 ml Erlenmyer shake flask containing 150 ml of each medium and incubated on a rotary shaker at 27 °C.

Isolation and detection of the toxins

After incubation, the culture filtrate (100 ml) was adjusted to pH 3.0 with 1 N HCl and extracted with the same volume of ethyl acetate. After the extract was washed with 5% $NaHCO_3$, it was concentrated *in vacuo*. Crude paecilotoxin fraction thus prepared was dissolved in a small amount of methanol. Each sample was passed through a FH type membrane filter (Nihon Millipore, Tokyo, Japan) and used for high performance liquid chromatography (HPLC) analysis.

Paecilotoxins were detected and determined by the following method. Since they have antimicrobial activity against various gram-positive bacteria, paecilotoxin production was checked by a bioassay method using *Bacillus subtilis* PCI as a test organism. When the ethyl acetate extracts showed positive antimicrobial activity, paecilotoxins in each extract were determined by HPLC and compared with those of the standard paecilotoxins. The HPLC system used employed

Table 1.

Strains of *Paecilomyces lilacinus* used in this study and their origin.

CBS 346.48 = IMI 27,830 = ATCC 10114, ex type of *Paecilomyces lilacinus*, isolated from soil, Ithaca, USA
CBS 429 = RS 410, ex Diaprepes abbreviatus (Coleoptera, Curculionidae), Florida, USA
CBS 430.87 = RS 986, ex Bombys mori (Lepidoptera, Bombycidae), Suginami, Japan
CBS = 431.87 = RS 2182, ex egg of mass root knot nematode, Philipines
CBS 432.87 = RS 2183, ex egg of mass root knot of nematode, Peru
CBS 226.73, ex intestine of Chameleo delepsi, Utrecht, the Netherlands
CBS 940.73, ex Aethus sp. (Coleoptera), Tago, Ghana
M5218 (Toyo Jozo Co., Ltd., Japan), ex soil, Japan
4-DS-35-4 (National Institude of Hygienic Science, Tokyo Japan), ex soil, Nagasaki, Japan
IFM 40621, ex soil
IFM 40624 (Ashigara Hospital, Japan), ex human eye as a paecilomycosis
Odashima (School of Medicine, Tokai University, Japan), ex human skin as paecilomycosis
SANK 10380 = ATCC 10114 (see CBS 346.48 strain)
IAM 7002, ex soil, Japan
C-37430 = IFM 4113, ex soil, Japan
A-267 = IFM A267, ex soil (see ref. 2)
C-12, a mutant from A-267, requiring leucine for growth
KU 0048, ex human eye, as a paecilomycosis
KU 0064, ex human eye, as a paecilomycosis
PL624 = IFM PL624, ex soil

IFM: Research Center for Pathogenic Fungi and Microbial Toxicoses, China University, Japan.
IAM: Institute of Applied Microbiology, University of Tokyo, Japan.
KU: Faculty of Medicine, Kyushu University, Japan.
CBS: Centraalbureau voor Schimmelcultures, The Netherlands.
SANK: Sankyo Co., Ltd., Japan.

a BIP-1 pump and a UVIDEC-100-IV variable-wavelength UV detector, all from Jasco, Tokyo, Japan.

All chromatograms were further obtained by the systems of packing: Cosmosil $5C_{18}$ (4.6 × 150 mm, Nakarai Chemicals, Japan); solvent conditions: methanol : 2-propanol : water : — acetonitrile : diethylamine (40 : 30 : 20 : 10 : 0.1); flow rate: 1.5 ml/min. Crystalline paecilotoxins A, B and C were prepared in our laboratory and used as standards.

Table 2. Time course of paecilotoxin production in three different media.

	Production of paecilotoxins* (positive strains/tested strains)		
	at 5 day	at 8 day	at 15 day
Medium A (pH 4.2 to 4.8)	7/20	14/20	15/20
Medium B (pH 8.2 to 8.9)	10/20	18/20	19/20
Medium C (pH 6.5 to 7.2)	6/20	15/20	17/20

* Paecilotoxin production was checked by bioassay method using *B. subtilis*. A clear inhibition zone around a paper disk (7 mm in diameter) with ethyl acetate extracts was considered to indicate the production of paecilotoxins.

Results and discussion

Twenty strains of *P. lilacinus* from various culture collections were cultured in three different media. At 5, 8 and 15 days after the inoculation, antimicrobial activities of the culture extract were determined respectively and the results are shown in Table 2. About 40% of the strains showed antimicrobial activity in all media after 5 days of culture. Rate of the toxin producing-strains increased with incubation time and after 15 days of culture most of the strains tested produced the toxins. These data indicate that an alkaline medium is more appropriate than a neutral or acid one for the production of paecilotoxins. *P. lilacinus* also seems to have alkalophilic or alkalo-resistant nature. These may be the reasons that this strain has frequently been isolated using an alkaline medium. The culture extracts which showed antimicrobial activity were further analyzed by HPLC and studies showed that all extracts with antimicrobial activity have the three paecilotoxins, A, B and C. A typical HPLC pattern of paecilotoxins produced by the strain 'Odashima' which was isolated from cutaneous paecilomycosis is shown in Fig. 2.

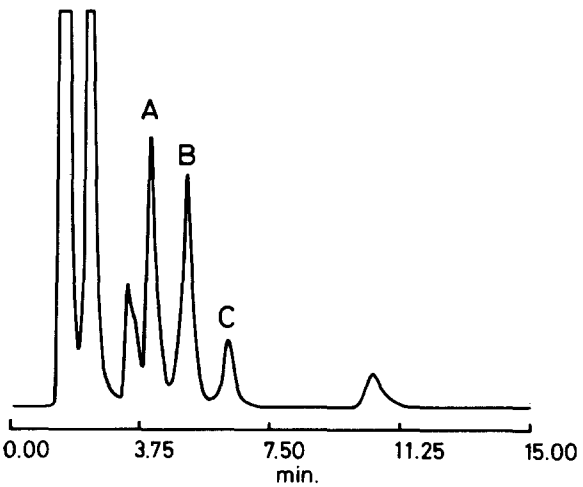


Fig. 2. HPLC pattern of paecilotoxins from ethyl acetate extract of the strain of *Paecilomyces lilacinus* (Odashima). A, B and C indicate each toxin peak and min indicates retention time.

Because *P. lilacinus* not only occurs as a soil saprophyte, but is also pathogenic to insects, nematodes and humans, it is striking that the different isolates all produce paecilotoxins. Although there are some minor morphological differences (*e.g.* entomopathogenic strains often produce synnemata on the insect host) in the strains of various habitat, the production of paecilotoxins indicates that these strains indeed belong to the same taxon. In addition, the paecilotoxins may play an important role not only of the infection process of mycoses in human case, but also with insects and nematodes.

Another active substance was detected on the mutant strain (C-12) which was derived from the strain A-269 in our laboratory. This substance was found to accumulate in the culture medium and it was purified and finally crystallized. Structural studies by NMR and mass spectral revealed the structure to be 2-isopropyl malic acid($C_7H_{12}O_5$). This substance is known to be a direct biosynthetic precursor of leucine. Therefore, the mutant strain might be devoid of an enzyme, 2-isopropyl malic acid isomerase (E.C. 4.2.1.33). Paecilotoxins have a high leucine content; therefore, the mutant may be a good tool for further analysis on this biosynthetic pathway.

Since paecilotoxin production was confirmed in most strains of *P. lilacinus* except for the strain RS-2182, this character might be used as chemotaxonomical index for this species. Therefore, further testing of toxin production using a number of strains as well as optimization studies of the toxin production are interesting. Our preliminary studies indicate that this exceptional strain RS-2182 produces other active substances against *B. subtilis*. Isolation studies are now in progress in our laboratory.

Structures of paecilotoxins E and F as a minor metabolites have recently been reported [5, 6]. In 1987, Radics *et al.* [7] reported the isolation of two new paecilotoxins H and K from another *Paecilomyces* species, *P. marquandii* (Massee) Hughes. Our comparative studies indicated that paecilotoxins H and K are respectively identical with paecilotoxins E and F [8]. *P. marquandii* and *P. lilacinus* are very related species. *P. marquandii* is also a soil-borne fungus and it seems that it is restricted to this habitat in contrast with *P. lilacinus* which also occurs on insects, nematodes, humans and animals. Therefore, present results may indicate that both species are biochemically related.

Further studies on the paecilotoxin production from the other species of *Paecilomyces* [2, 9] are interesting from the viewpoint of chemotaxonomy of the genus *Paecilomyces*.

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Address for offprints: Yuzuru Mikami, Department of Experimental Chemotherapy, Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1, Inohana, Chiba, Japan (280).