

Argifin, a New Chitinase Inhibitor, Produced by *Gliocladium* sp. FTD-0668

I. Taxonomy, Fermentation, and Biological Activities

SATOSHI ŌMURA,* NORIKO ARAI, YUUICHI YAMAGUCHI, ROKURO MASUMA, YUZURU IWAI,
MICHIO NAMIKOSHI†, ANDREAS TURBERG,†† HEINZ KÖLBL†† and KAZURO SHIOMI

Research Center for Biological Function, The Kitasato Institute,
5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan

† Department of Ocean Sciences, Tokyo University of Fisheries
4-5-7, Konan, Minato-ku, Tokyo 108-8477, Japan

†† Bayer AG, Biological Technology Center, Business Group Animal Health,
D-51368 Leverkusen, Germany

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A new chitinase inhibitor, named argifin, was isolated from the cultured broth of a fungal strain FTD-0668. The strain was identified as *Gliocladium* sp. from morphological characteristics. The IC_{50} value of argifin against *Lucilia cuprina* chitinase was $3.7 \mu\text{M}$. Argifin arrested the moult of cockroach larvae upon injection into the ventral abdominal part.

Chitin occurs in fungi, some algae, and many invertebrates including insects, but is not found in vertebrates. Thus chitin synthesis and degradation might be expected to be specific targets for fungicides and insecticides.^{1,2)} Though chitin synthesis inhibitors are practically used such as fungicidal polyoxins and insecticidal benzoylphenylureas, chitin degradation inhibitors have not yet been used commercially. Chitinase (EC 3.2.1.14) hydrolyzes chitin into oligomer of *N*-acetylglucosamine, and only few inhibitors have been reported, e.g. allosamidin,³⁾ styloguanidine,⁴⁾ and cyclo-(L-arginyl-D-prolyl).⁵⁾ An inhibitor of chitinase would be expected to inhibit moulting of insects and prevent their maturation to the adult reproductive stage.

In the course of our screening for new insecticides, we have found a new cyclic peptide chitinase inhibitor, named argifin (Fig. 1), from the cultured broth of *Gliocladium* sp. FTD-0668.⁶⁾ In this paper, we report the taxonomy of the producing organism together with fermentation and biological properties of argifin. Isolation, physico-chemical properties, and structure elucidation of argifin will be described in the accompanying paper.⁷⁾

Materials and Methods

Chemicals

Chitinase from *Streptomyces griseus*, 4-methylumbelliferone, and 4-methylumbelliferyl- β -D-*N,N',N''*-triacetylchitotriose were obtained from Sigma Chemical Co. Chitinase from *Vibrio alginolyticus* was obtained from Kyowa Medex Co.

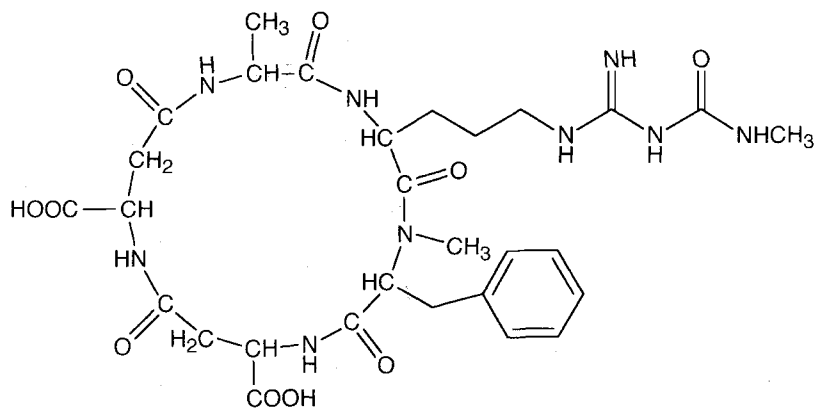
Media

The seed medium was composed of glucose 2.0%, Polypepton (Nihon Pharmaceutical Co.) 0.5%, agar 0.1%, yeast extract (Oriental Yeast Co.) 0.2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, and KH_2PO_4 0.1%. The pH was adjusted to 6.0 prior to sterilization. The production medium was composed of sucrose 2.0%, glucose 1.0%, corn steep powder (Shono Starch Co.) 0.5%, meat extract (Kyokuto Pharmaceutical Industrial Co.) 0.5%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, CaCO_3 0.3%, agar 0.1%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001%, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.001%, ZnSO_4 0.001%, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.001%, and $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$ 0.001%. The pH was adjusted to 6.0 prior to sterilization.

Preparation of Chitinase from *Lucilia cuprina*

Crude chitinase solution of blowfly, *Lucilia cuprina*, was

Fig. 1. Structure of argifin.



prepared as follows. Forty late pupae of *L. cuprina* were homogenized in 8 ml of 100 mM sodium phosphate buffer (pH 7.0) in a Teflon-glass potter. The homogenate was filtered through a double layer of cheese cloth. The filtrate was subsequently treated with ultrasonics (3×5 seconds, 50W), and then centrifuged for 1 hour, at 100,000 *g*, 4°C. Aliquots of the supernatant were diluted to *ca.* 7 mg protein/ml with the same buffer and stored at -20°C. The protein solution was diluted to an appropriate concentration before use.

Assay for Chitinase Inhibitory Activity

10 μ l of the sample solution was put into each well of 96-well black plate (Fluoro B plate, Dainippon Pharmaceutical Co.) and the plate was dried *in vacuo*. Next 20 μ l of 100 mM sodium phosphate buffer, pH 7.0, was added to each well and the plates were shaken on a microplate mixer. Then 30 μ l of diluted chitinase solution (containing 4 μ g protein for 37°C assay and 15 μ g protein for 20°C assay) was added to each well. The plate was put into a fluorometer (Fluoroscan II, Labsystems) and preincubated at 37°C for 5 minutes. Then 50 μ l of 80 μ M 4-methylumbelliferyl- β -D-N,N',N''-triacetylchitotriose in the buffer was added to each well, and the reaction initiated. The incubation was carried out at 37°C for 10 minutes or 20°C for 15 minutes. Fluorescence (excitation at 355 nm, emission at 460 nm) was measured at intervals of 80 seconds. The rate of 4-methylumbelliferone production was corrected by calibrating the quenching ratio of each sample using the mixture of the sample and 4-methylumbelliferone. For argifin, no significant quenching was determined at the excitation and emission wavelength.

Assay for Antimicrobial Activity

The antimicrobial spectrum of argifin was determined using i.d. 6×1.5 mm paper discs (Advantec Toyo Kaisha). Antimicrobial activity was observed after 20 hours of incubation at 37°C for bacteria except *Xanthomonas campestris* pv. *oryzae* or longer incubation at 27°C for fungi or yeasts. *X. campestris* pv. *oryzae* was incubated for 20 hours at 27°C.

Assay for Cockroach Larvae Injection

20 μ g of Argifin dissolved in 1 μ l distilled water was injected in the ventral abdominal region of second instar larvae of *Periplaneta americana* (american cockroach) or third instar larvae of *Blattella germanica* (german cockroach) using a Hamilton micro syringe in three separate trials (8~20 larvae/trial) and compared to mock-injected (1 μ l water) controls. Cockroach larvae were put in a petri dish containing a filter paper and a piece of wafer. Petri dishes were placed in a humidified incubator at 28°C and 75% relative humidity. Mortality and status of development were monitored after 24 hours, 5 days, and 20 or 23 days and compared to control mortality.

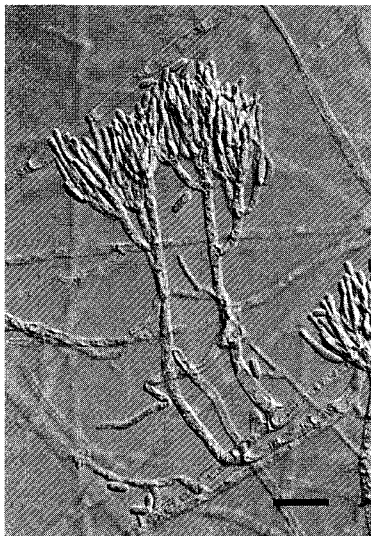
Results

Taxonomy of the Producing Strain FTD-0668

The producing fungal strain was isolated from a soil sample collected in Ponape Island, Federated State of Micronesia. Morphological properties were examined after incubation at 25°C for 14 days on potato dextrose agar (Difco), malt extract agar (malt extract 2.0%, peptone

Fig. 2. Photomicrograph of penicillate and phialoconidia of strain FTD-0668.

Bar represents 20 μm .



0.1%, glucose 2.0%, and agar 2.0%), corn meal agar (Difco), and Miura's medium (glycerol 0.1%, KH_2PO_4 0.08%, K_2HPO_4 0.02%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02%, KCl 0.02%, NaNO_3 0.2%, yeast extract 0.02%, and agar 1.5%). The strain grew moderately to form white to light yellow colonies with a diameter of 40~60 mm. Reverse of the colonies was white to pale yellow. The colonies were floccose on each media. Under microscopic observation (Fig. 2), conidiophores were erect or branched and 50~70 μm long, and their upper portions bare penicillate branches. Phialides that composed of penicillate branches were cylindrical to obclavate and $16\sim 22 \times 1.5\sim 2.0$ μm in size. Phialoconidia were cylindrical to ellipsoidal, $5.0\sim 7.0 \times 2.0\sim 2.5$ μm in size, and forming mucilaginous droplets.

From the above characteristics, strain FTD-0668 was considered to belong to genus *Gliocladium* and named *Gliocladium* sp. FTD-0668. The culture was deposited at Fermentation Research Institute, Agency of Industrial Science and Technology Japan, as FERM P-16626.

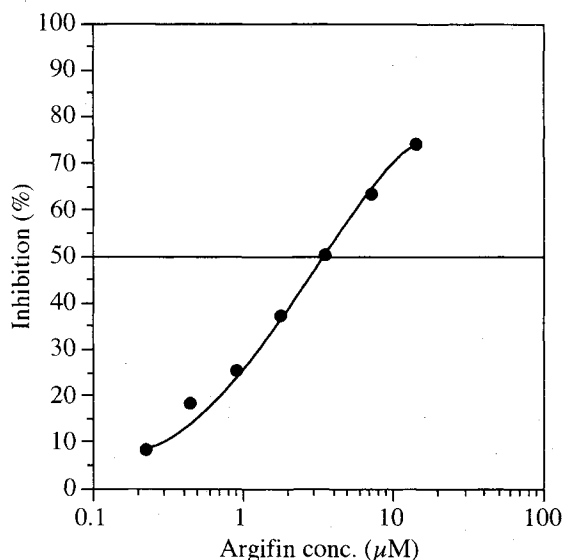
Fermentation of Argifin

A stock culture of strain FTD-0668 grown on potato dextrose agar was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium. It was incubated on a rotary shaker (210 rpm) at 27°C for 72 hours. One ml

Table 1. *Lucilia cuprina* chitinase inhibition by argifin and allosamidin.

Compound	IC_{50} (μM)	
	37°C	20°C
Argifin	3.7	0.10
Allosamidin	0.0023	0.00040

Fig. 3. Inhibition of *Lucilia cuprina* chitinase by argifin.



of the seed culture was transferred into each of eighty 500-ml Erlenmeyer flasks containing 100 ml of a production medium. The fermentation was carried out on the rotary shaker at 27°C for 168 hours. The production of argifin started at day 4 and reached maximum at day 7. Argifin was produced mainly in mycelia.

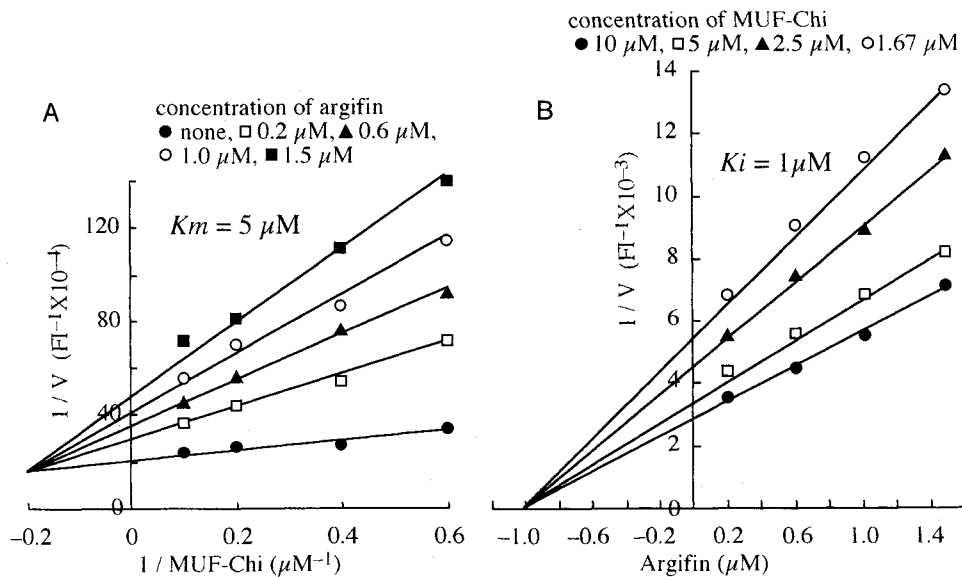
Biological Activities of Argifin

Chitinase Inhibitory Activity

Chitinolytic activity of chitinase from *Lucilia cuprina* was studied in the presence of argifin and allosamidin,³⁾ a known potent inhibitor of chitinase (Table 1). Argifin inhibited the chitinase in a dose-dependent manner (Fig. 3) with IC_{50} values of 3.7 μM at 37°C and 0.10 μM at 20°C. Allosamidin showed much potent inhibition with IC_{50}

Fig. 4. Kinetic analyses of chitinase inhibition by argifin.

A: Lineweaver-Burk plot. B: Dixon plot.



values of 2.3 nM at 37°C and 0.40 nM at 20°C. Argifin also inhibited chitinases from *Streptomyces griseus* and *Vibrio alginolyticus* with IC_{50} values of 14.8 μM and 0.9 μM at 37°C, respectively. Though argifin showed weaker inhibition than allosamidin, it is the first chitinase inhibitor structurally not related to allosamidin (non-sugar inhibitor) that has been reported to show inhibition in a submicromolar range.

The IC_{50} ratio (measured at 37°C and 20°C) was forty in argifin, compared to allosamidin of about six. This result may be due to the mechanism of inhibition. The preliminary kinetic analyses of argifin were performed using chitinase from *L. cuprina* at 37°C. The Lineweaver-Burk plot showed mixed inhibition with the K_m value of 5 μM (Fig. 4A), and the Dixon plot showed the K_i value of 1 μM (Fig. 4B). Allosamidin was reported to show competitive inhibition against *Bombyx mori* chitinase.³⁾

Antimicrobial Activities

Argifin did not inhibit the growth of the following microorganisms at 10 μg /disc: *Bacillus subtilis* PCI 219, *Micrococcus luteus* PCI 1001, *Mycobacterium smegmatis* ATCC 607, *Staphylococcus aureus* FDA 209P, *Acholeplasma laidlawii* PG 8, *Bacteroides fragilis* ATCC 23745, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* P3, *Xanthomonas campestris* pv. *oryzae* KB 88, *Candida*

albicans KF 1, *Saccharomyces cerevisiae* KF 26, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581, and *Pyricularia oryzae* KF 180.

Cytotoxicities

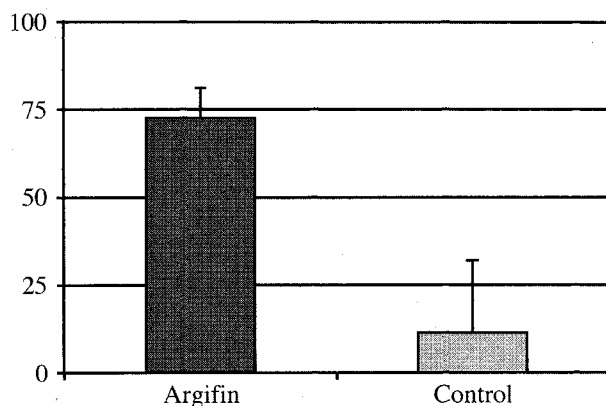
Argifin did not inhibit the growth of P388 and HL-60 cells at 25 μg /ml.

Cockroach Larvae Mortality by Injection Assay

Argifin (20 μg) was injected into cockroach larvae in three separate trials and compared to mock injected controls. Mortality was assessed at 1, 5, and 20 or 23 days post injection, and final values are shown in Fig. 5. Three separate trials repeatedly showed the efficacy of argifin against larval stages of *Periplaneta americana* (american cockroach) and *Blattella germanica* (german cockroach). Argifin showed 73% mortality of cockroaches, while control mortality was only 12%. There was an exceptionally high control mortality in the initial phase of the experiment. No control mortality was observed after day 1.

Most of the dead cockroach larvae showed no signs of moulting. Cockroach larvae killed during moulting showed new cuticle formation below the partially opened old cuticle and were unable to leave the old exuvia leading to their death shortly after sclerotization of the new cuticle. Fig. 6A shows normal moulting of *P. americana* second instar

Fig. 5. Efficacy of argifin against cockroach larvae.



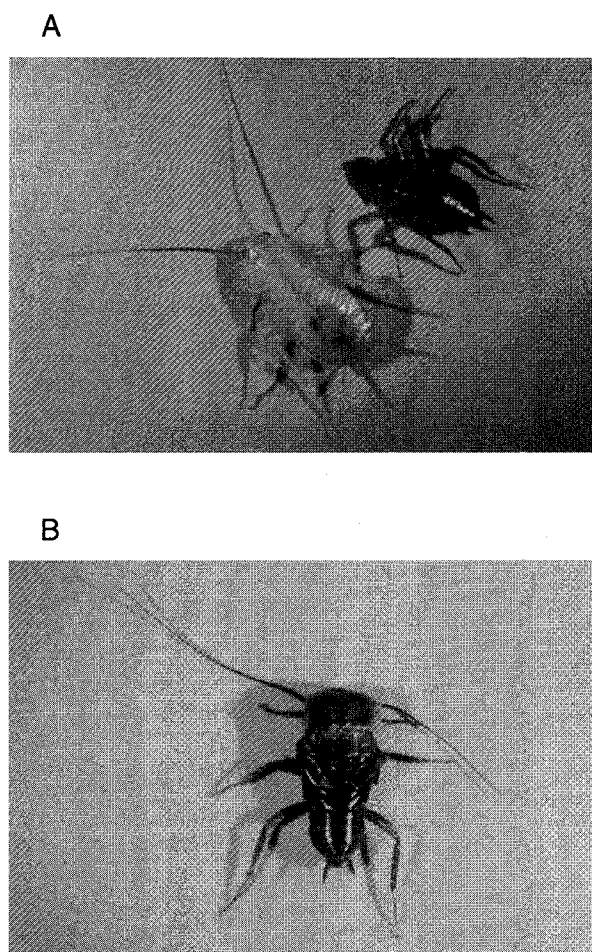
20 μg of Argifin dissolved in 1 μl distilled water was injected into the second instar larvae of *P. americana* or the third instar larvae of *B. germanica* using in three separate trials (8~20 larvae/trial) and compared to mock-injected (1 μl water) controls. Mortality was assessed after 20 or 23 days.

larva. White larva is a freshly emerged larva, and an exuvia is shown right. Fig. 6B shows the second instar larva arrested during moult by injection of argifin. A part of the old cuticle along the dorsal line of the larva has opened in the effort of the larva to emerge. The formation of new cuticle of the moulting larva has stopped and sclerotization already occurred. The head capsule and abdomen were not able to move from the exuvia of the old larva. The cockroach larva showed ongoing efforts to leave the old cuticle by increasing of the hemolymph pressure during its pre-sclerotization phase. With onset of sclerotization of the new cuticle the larva was trapped in the old cuticle and died.

Discussion

We have isolated a new chitinase inhibitor, argifin, from the culture broth of *Gliocladium* sp. FTD-0668. It was the first chitinase inhibitor produced by fungi. SAKUDA *et al.* have demonstrated that pseudooligosaccharide chitinase inhibitors, allosamidins, from *Streptomyces* sp. are competitive inhibitors.³⁾ Conversely, argifin showed mixed inhibition, and the structure is not related to saccharides. Therefore, the inhibition mechanism of argifin is arguably different from that of allosamidin.

Fig. 6. Moulting of *P. americana* second instar larva and its inhibition by argifin.



A: Normal moult of *P. americana* second instar larva. White larva is a freshly emerged larva, and an exuvia is shown right. B: The second instar larva arrested during moult by injection of argifin (20 μg).

It was also shown that argifin was capable of acting *in vivo* when injected into cockroach larvae. As an important prerequisite of the moulting process, a moulting gap has to be formed.⁸⁾ This occurs by detaching of the epidermal cells from the old endocuticle, a process called apolysis. The gap is filled with exuvial fluid containing digestive enzymes to degrade the non-sclerotized endocuticle layer of the old cuticle (exuvia). One of the key enzymes involved in this process is a chitinase digesting the polymeric chitin chains to oligomers and finally down to dimers of *N*-acetylglucosamine. Hydrolysis to *N*-acetylglucosamine monomers is catalyzed by β -*N*-acetylglucosaminidase.⁹⁾

Symptomatology of cockroach larvae injection bioassay

indicates the possibility of failure in moult in individuals injected at the onset of moulting stage. Apolysis seemed to be incomplete and the new larval instar failed to emerge from its exuva. Further studies will be required including electron microscopic investigations of the integument and epidermal parts of the cockroach larvae during moult.

Chitinase is widely distributed in the group of bacteria, fungi, plants, and arthropods, but it is not essential for mammals. Chitinase inhibitors would be expected to interfere with the life cycle of the former group, but not that of the latter. Therefore, argifin is an interesting compound as a lead for the development of novel insecticide.

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