CYCLOOCTATIN, A NEW INHIBITOR OF LYSOPHOSPHOLIPASE, PRODUCED BY *Streptomyces melanosporofaciens* MI614-43F2

TAXONOMY, PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

TAKAAKI AOYAGI[†,††], TAKAYUKI AOYAMA[†], FUKIKO KOJIMA[†], SEIKO HATTORI[†], YOSHIKO HONMA[†], MASA HAMADA[†] and TOMIO TAKEUCHI[†]

[†]Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan
[††]Showa College of Pharmaceutical Sciences, Machida-city, Tokyo 194, Japan

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Cyclooctatin has been isolated from *Streptomyces melanosporofaciens* MI614-43F2 as part of a program designed to find microorganism-produced inhibitors of lysophospholipase. It was purified by chromatography on silica gel, Capcell Pak C_{18} (HPLC) and Sephadex LH-20 followed by solvent extraction and then isolated as a colorless powder. Cyclooctatin has the molecular formula of $C_{20}H_{34}O_3$. It is competitive with the substrate, and the inhibition constant (*Ki*) was 4.8×10^{-6} M.

Lysophospholipase [EC 3.1.1.5] (Lyso-PL) catalyzes the hydrolysis of the fatty acid ester bonds of lysophospholipids liberating the corresponding free fatty acid and glycerophosphate. Prominent blood or tissue accumulations of eosinophilic leukocytes occur in many allergic reactions and inflammatory diseases. Of the human peripheral blood leukocytes, sonicates of eosinophils express 3 to 8 times as much Lyso-PL activity as do sonicates of comparable numbers of neutrophils or mononuclear leukocytes. This enzyme appears to be the sole component of Charcot-Leyden crystals (CLC) that are observed in human tissues and fluids usually in association with eosinophilic inflammatory reactions. Thus, the CLC as a repository of human eosinophil Lyso-PL may posses a biological function more significant than being solely a hallmark of an eosinophilic inflammatory process[1,2].

As reported in this paper, we searched for new Lyso-PL inhibitors in culture broths of microorganisms. In this communication we report the taxonomy, production, isolation, physico-chemical properties and biological activities of the inhibitor cyclooctatin.

Materials and Methods

Chemicals

Chemicals employed were as follows: Silica gel 60 and TLC-plate Silica gel F_{254} (0.25 mm thickness) from E. Merck, Darmstadt, FRG; packed column of Capcell Pak C_{18} from Shiseido Co., Tokyo, Japan; 1-palmitoyl lysophosphatidylcholine (LysoPC) from Sigma Chemical Co., St Louis, U.S.A.; LysoPC-[palmitoyl-1-^{14}C] and a scintillator Aquasol-2 from New England Nuclear, Boston, U.S.A. All other chemicals were of analytical grade.

Enzymes

Lyso-PL was prepared from bovine liver as described by JONG *et al.*[3]. Partially purified enzyme was used in this assay.

Microorganism

Strain MI614-43F2 was isolated from a soil sample collected in Zentsugi, Kagawa Prefecture, Japan and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession number FERM P-10431.

Taxonomic Characterization

Morphological and physiological properties of the strain were examined according to SHIRLING and GOTTLIEB[4]; several other tests were also used.

Production of Cyclooctatin

The strain MI614-43F2 was inoculated into 110 ml of a production medium consisting of galactose 2.0%, dextrin 2.0%, glycerol 1.0%, Bacto-Soytone (Difco Lab.) 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% (pH 7.4) in a 500-ml Erlenmeyer flask, and cultured at 27°C for 3 days on a rotary shaker (180 rpm). Two ml of the above seed culture was transferred to 110 ml of the same medium in a 500-ml Erlenmeyer flask and cultured for 3 days under the same conditions.

Isolation of Cyclooctatin

The culture broth was filtered and the culture filtrate was extracted with an equal volume of BuOAc. The active extract was concentrated to dryness under reduced pressure. The dried material was dissolved in a small volume of $CHCl_3$, charged to a silica gel column ($\times 50$ w/w powder) and eluted with $CHCl_3$ - MeOH (95 : 5). The active fractions were collected and evaporated to give a brownish syrup. The syrup was suspended in a solvent mixture of $CHCl_3$ - MeOH - AcOH (60 : 35 : 5). The suspension was passed through a column of silica gel ($\times 50$ w/w powder) which had been packed with the same solvent mixture. The eluate was concentrated under reduced pressure to give brownish powder containing cyclooctatin. The crude powder was further purified by a reversed phase HPLC using a Capcell Pak C_{18} column (2.0 × 25 cm, flow rate 8 ml/minute, using Waters ALP/GPC 200 system) with 50% aqueous CH_3CN. The eluate fractions containing cyclooctatin were concentrated under reduced pressure to give a pale brownish powder. This powder was dissolved in a small volume of MeOH and the solution was subjected to Sephadex LH-20 column (1.8 × 110 cm) chromatography developed with MeOH. The eluate was concentrated under reduced pressure to give cyclooctatin as a colorless powder.

Assay for Phospholipase (PL) and Inhibitory Activity

Lyso-PL activity was measured by a modification of the method of JONG *et al.*[3]. The reaction mixture (total 0.5 ml) consisted of 20 mM potassium phosphate buffer (pH 7.5), 400 μM LysoPC, 0.36 μM LysoPC-[palmitoyl-1-^{14}C] (2×10^4 dpm), Lyso-PL and water or aqueous solution containing the test compound. The enzyme reaction was started by the addition of the enzyme, following incubation at 37°C for 60 minutes and stopped by the addition of a solvent mixture (1.0 ml) of iso-PrOH - *n*-heptane - 0.5 N H_2SO_4 (40 : 10 : 1). After mixing for 10 seconds with a Vortex-Genie mixer and centrifugation at 1,300 × *g* for 5 minutes, the radioactivity of upper layer (100 μl) was counted with a scintillator (Aquasol-2, 5 ml).

PL-A_2, PL-C and PL-D activities were measured as reported previously[5].

The percent inhibition was calculated by the formula $(A-B)/A \times 100$, where A is dpm of liberated radioactive product by the enzyme in the system without an inhibitor and B is that with an inhibitor. IC_{50} value shows the concentration of inhibitor at 50% inhibition of enzyme activity.

Physico-chemical Properties

Melting point was taken using a Yanaco MP-S3 apparatus and was uncorrected. UV spectrum was recorded on a Hitachi U-3210 spectrophotometer and IR spectrum on a Hitachi 260-10 spectrophotometer. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. Mass spectrum was obtained on a Hitachi M-80 mass spectrometer.

Results and Discussion

Taxonomic Characterization of the Producing Strain

Strain MI614-43F2 has branched substrate mycelia, from which aerial hyphae develop in the form of open spirals. No whirl-formation was observed. Matured spore-chains usually bear more than 10 conical spores. Spores ranged about 0.8~1.0 by 1.0~1.2 μm in size and have a rugose surface. Aerial mass color of the colony was yellowish gray to light gray and was gradually glistening with dark gray. The color of vegetative growth was colorless to yellowish brown. Melanoid pigments were none to faintly brown with red.

The whole-cell hydrolysate of the strain showed that it contained LL-diaminopimelic acid. Based on its characteristics, strain MI614-43F2 is considered to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *Streptomyces melanosporofaciens* and *Streptomyces hygroscopicus* are recognized to be similar to the strain MI614-43F2. The results of comparison of the strain MI614-43F2 and these two species are summarized in Table 1. As will be apparent from Table 1, the strain MI614-43F2 is closely related to *S. melanosporofaciens* and *S. hygroscopicus* and in their microbiological properties except for

Table 1. Comparison of taxonomic characteristics of strain MI614-43F2 with *Streptomyces melanosporofaciens* and *Streptomyces hygroscopicus*.

	MI614-43F2 (FERM P-10431)	*S. melanosporofaciens* IMC. S-0312 (ISP 5318)	*S. hygroscopicus* IMC. S-0748 (ISP 5578)
Spore chain morphology	Spirals	Spirals	Spirals
Spore surface	Rugose	Rugose	Rugose
Aerial mass color	Yellowish gray to light gray	Yellowish gray to light gray	Grayish white to light gray
Glistening of aerial hyphae	Positive	Positive	Positive
Color of vegetative growth	Colorless to yellowish brown	Colorless to yellowish brown	Colorless to yellowish brown
Soluble pigment	Negative	Negative	Negative
Melanin formation:			
ISP-medium 1	Negative	Negative	Negative
ISP-medium 6	Negative	Negative	Negative
ISP-medium 7	Negative (faintly brown with red)	Negative (faintly brown with red)	Negative
Hydrolysis of starch	Positive	Positive	Positive
Coagulation of skim milk	Positive	Positive	Negative
Peptonization of skim milk	Positive	Positive	Positive
Liquefaction of gelatin:			
Plain gelatin	Positive	Positive	Positive
Glucose-peptone-gelatin	Positive	Positive	Positive
Nitrate reduction	Positive	Positive	Positive
Carbon utilization:			
D-Glucose	+	+	+
L-Arabinose	+	(+)	±
D-Xylose	+	+	(+)
D-Fructose	+	+	+
Sucrose	+	−	−
Inositol	+	+	−
L-Rhamnose	+	+	+
Raffinose	+	+	−
D-Mannitol	+	+	+
Lactose	+	+	(+)

+, Utilization; ±, doubtful utilization; −, no utilization.

the utilization of sucrose. Strain MI614-43F2 is different from *S. hygroscopicus* in coagulation of skim milk, utilization of inositol and raffinose, melanoid pigments and color characteristics of aerial hyphae. Therefore, we estimated that the strain MI614-43F2 belongs to *S. melanosporofaciens*, and it was designated as *S. melanosporofaciens* MI614-43F2.

Production and Isolation of Cyclooctatin

The strain of *S. melanosporofaciens* MI614-43F2 was cultured in Erlenmeyer flasks at 27°C for 3 days on a rotary shaker. The time course of the production is shown in Fig. 1. The maximum peak of cyclooctatin production in the flasks was obtained at 3 days, thereafter the production slowly decreased. From the culture filtrate (39 liters), cyclooctatin was isolated as shown in Fig. 2. The total yield of cyclooctatin was 13.6 mg. The purity of each preparation was confirmed by TLC and HPLC.

Fig. 1. Time course of cyclooctatin production by *Streptomyces melanosporofaciens* MI614-43F2.

● Inhibition, ○ pH, ▲ galactose, △ glycerol.

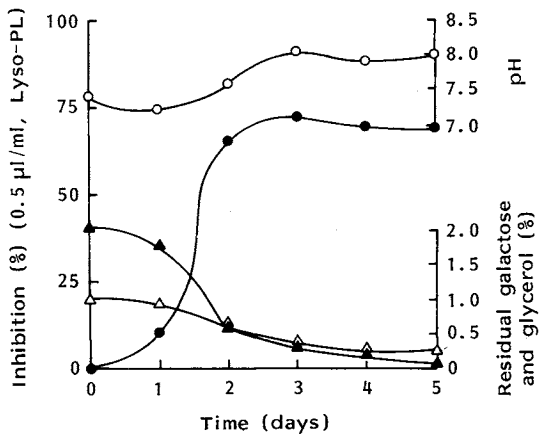


Fig. 2. Isolation of cyclooctatin.

S. melanosporofaciens MI614-43F2

27°C, 72 hours

Mycelia

Culture filtrate (39 liters, IC_{50} = 1.1 µl/ml)

BuOAc extraction

Silica gel chromatography

$CHCl_3$ - MeOH (95 : 5)

Silica gel chromatography

$CHCl_3$ - EtOAc - AcOH (60 : 35 : 5)

HPLC (Capcell pak C_{18})

50% aq CH_3CN

Sephadex LH-20

MeOH

Cyclooctatin (13.6 mg, IC_{50} = 2.9 µg/ml)

Table 2. Physico-chemical properties of cyclooctatin.

Appearance	Colorless powder
MP	183~185°C (dec.)
$[\alpha]_D^{27}$ (*c* 0.5, MeOH)	+90.6°
FD-MS (*m/z*)	322 (M^+)
Elemental analysis	
Found:	C 74.09, H 10.63
Calcd for $C_{20}H_{34}O_3$:	C 74.45, H 10.63
UV (EtOH)	End absorption
IR ν_{max}^{KBr} cm^{-1}	3430, 2960, 1636, 1385, 1178, 1028
Rf value on TLC	0.47 ($CHCl_3$ - MeOH = 9 : 1, silica gel)
Color reaction	Mo-H_2SO_4
Solubility	
Soluble:	DMSO, MeOH, Me_2CO, EtOAc
Insoluble:	H_2O

Table 3. Inhibitory activities of cyclooctatin against phospholipases.

	IC_{50} (µg/ml)			
	Lyso-PL	PL-A_2	PL-C	PL-D
Cyclooctatin	2.9	65.0	70.0	>100
Terpentecin	16.5	>100	>100	>100
Plipastatin A_1	>100	4.2	1.9	2.0
EDDS	>100	>100	3.8	19.0

EDDS; (*S,S*)-*N,N'*-ethylenediaminedisuccinic acid.

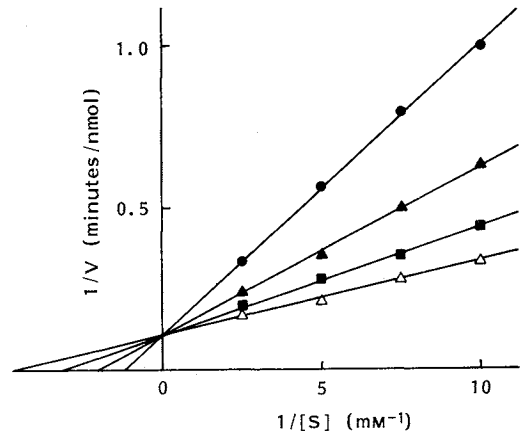
Physico-chemical Properties of Cyclooctatin

The physico-chemical properties of cyclooctatin are summarized in Table 2. The molecular weight and formula were determined to be $C_{20}H_{34}O_3$ (MW 322.49) by FD-MS and elemental analysis. Cyclooctatin is soluble in methanol, ethyl acetate and dimethyl sulfoxide, but insoluble in water.

Determination of the structure of cyclooctatin will be described in the following paper[6].

Fig. 3. Lineweaver-Burk plot of inhibition of Lyso-PL by cyclooctatin.

● I=5.0 μg/ml, ▲ I=2.5 μg/ml, ■ I=1.25 μg/ml, △ I=0 μg/ml.



Biological Activities of Cyclooctatin

The inhibitory activities of cyclooctatin and various inhibitors of phospholipases are shown in Table 3. Terpentecin is a structurally related compound as a diterpenoid[7]. Plipastatin A_1[5] and (*S,S*)-*N,N'*-ethylenediaminedisuccinic acid (EDDS)[8] are inhibitors of PL-A_2 and PL-C, respectively. Cyclooctatin inhibits specifically Lyso-PL and slightly PL-A_2 and PL-C. As shown in Fig. 3, it is competitive with substrate. The *Ki* value of cyclooctatin is 4.8×10^{-6} M. It had no significant antimicrobial activity at 100 μg/ml. It has low toxicity; there were no deaths after ip injection of mice with 100 mg/kg.

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