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A Cytolytic Substance, Monopodialysin obtained from a *Streptomyces*. I. Isolation and Characterization of Monopodialysin produced by *Streptomyces aureomonopodiales*

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A intracellular cytolytic substance, named monopodialysin was isolated from the mycelium of a *Streptomyces* strain designated as *Streptomyces aureomonopodiales*. The cytolytic substance was purified by extraction with acetone, followed by column chromatography on silica gel and gel filtration on a column of Sephadex LH-20. Monopodialysin was obtained as colorless plate (M.W.<1000) and contained C, H, and O as elements. It showed very high hemolytic activity for several kinds of mammalian erythrocytes and its activity was inhibited by soy bean lecithin or cholesterol but no sucrose. Protoplasts of *Bacillus subtilis* and lysosomes from rat liver were also lysed but monopodialysin displayed no antimicrobial activity.

Keywords——*Streptomyces aureomonopodiales*; monopodialysin; cytolytic substance; hemolytic activity; lysosome lytic action; antimicrobial activity; protoplast lytic action; gel filtration

Several bacteria produce hemolytic toxins, for example Streptolysin S,[2)] Streptolysin O,[3)] Staphylococcal α-,[4)] β-,[5)] and Clostridium perfringens α-, θ-toxins,[6)] which are characterized as extracellular products of bacterial growth that are of relative large molecular size. In addition to the hemolytic action they cause gross lysis *in vitro* of one or more kinds of mammalian cells and most of them are proteins,[4)] some of which are considered to act as enzyme on cell membrane.[5,6)]

Apart from their possible importance as agents of pathogenesis, some of them exhibit remarkable specificity and when their nature and functions are better defined, they may prove to be useful reagents in the general study of biological membranes.

In the course of the screening of nonprotein and small molecular size hemolytic substance produced by *Streptomyces*, we found a cytolytic substance in the mycelium of Streptomyces strains.

A cytolytic substance named monopodialysin without antimicrobial activity was isolated from the mycelium of *Streptomyces* sp. F4810 designated as *Streptomyces aureomonopodiales*. The present paper is concerned with isolation and some properties of this cytolytic substance.

Experimental

The Source and Production of Cytolytic Principle——A strain of *Streptomyces* sp. F4810 found to produce hemolytic substance was classified as *Streptomyces aureomonopodiales*. This organism was grown in liquid medium of the following composition: 2.0% polypeptone, 0.2% yeast extract, 0.2% KH_2PO_4, 0.001% $MgSO_4$ (pH 7.0) for preparation of the inoculum. The inoculum was cultured in a 1 l shaking flask filled with 250 ml

1) Location: *133-1, Yamadakami, Suita-shi, Osaka 565, Japan.*
2) J. Koyama and F. Egami, *J. Biochem.*, **55**, 629 (1964).
3) J.E. Alouf and M. Raynaud, *Nature*, **196**, 374 (1962).
4) A.W. Bernheimer, *Science*, **159**, 847 (1968).
5) S.K. Maheswarum, K.L. Smith, and R.K. Lindorfer, *J. Bacteriol.*, **94**, 300 (1967).
6) S. Kadis and T.C. Montie, "Microbial Toxins," Vol. IIa, Academic Press, New York and London, 1971, p. 109.

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of the medium at 30° for 48—72 hr under shaking (125 rpm). A 10 ml sample of the resulting culture was transfered to a 1 l shaking flask containing 250 ml of the following medium: 0.2% potato starch, 1.0% dry yeast (Ebios), 1.0% pharmamedia (pH 6.0) and cultivated at 30° for 72 hr on reciprocal shaker (125 rpm). The hemolytic activities were assayed in the acetone extract of mycelium and culture filtrate.

Assay of the Hemolytic Activity——The various mammalian erythrocytes were washed with isotonic sodium phosphate buffer (115 mM Na_2HPO_4–146 mM NaH_2PO_4 8:2, pH 7.4) until the supernatant was colorless and diluted with the same buffer to give the suspension of the desired concentration (absorbance at 550 nm of 100% hemolysis caused by distilled water was between 0.6 and 0.7). To 3.0 ml of isotonic buffer was added 1.0 ml of the erythrocyte suspension and thoroughly mixed. The mixture was incubated at 37° for 30 min, cooled immediately in ice bath and then centrifuged at 3000 rpm for 10 min. Absorbance at 550 nm of the supernatant was determined. Monopodialysin and other reagents of low aqueous solubility, lecithin and cholesterol were dissolved in small amount of methanol or ethanol and added isotonic buffer to give a desired final concentration. One unit of hemolytic activity was defined as the concentration giving 50% hemolysis.

Determination of Lytic Activities on Bacterial Protoplast and Rat Liver Lysosome——*Bacillus subtilis* I.A.M. 1069 was cultivated in a medium containing NH_4Cl 2 g, Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 3 g, $MgCl_2$ 0.04 g, Na_2SO_4 0.11 g, casamino acid 2 g, glucose 10 g, tryptophan 0.02 g per liter of distilled water. After the bacterium was shake-cultivated at 37° for 5.5 hr, cell was harvested by centrifugation and suspended in a medium containing 0.5 M sucrose and culture components described above except for casamino acid, tryptophan and $MgCl_2$. To the bacterial suspension, 1.0 mg/ml lysozyme was added, incubating at 37° for 15 min. After observation of protoplast by phase microscopy, 1 mM $MgCl_2$ (final concentration) was added to give protoplast suspension. The protoplast suspension, 0.5 ml was added to 3.5 ml of the above culture medium containing the test sample. During shaking at 37° the decrease of absorbance at 550 nm was determined.

The lysosomal fraction was prepared from rat liver according to the method of Goldman *et al.*[7] The cytolytic activity was followed by measuring the release of acid phosphatase to the medium. Monopodialysin was dissolved in methanol. All incubation mixtures including the control contained 10 μl of methanol. The lysosomal suspensions were incubated for 15 min at 37°, cooled to 4°, centrifuged at 14500 ***g*** for 20 min and the resulting supernatants were assayed for acid phosphatase activity. *p*-Nitrophenyl phosphate 100 μl of 0.1 M was added to each sample (0.9 ml of supernatant) and the rate of *p*-nitrophenol release at 37°, was followed at 420 nm. Acid phosphatase activity in the supernatant is expressed as percentage of total activity obtained in the presence of 0.1% of Triton X-100. The values were corrected for rates obtained at zero time incubation.

Binding of Monopodialysin to Bovine Erythrocyte Ghost——Erythrocyte ghost was prepared by the method of Dodge *et al.*[8] About 5×10^8 ghost cells in isotonic phosphate buffer were preincubated with monopodialysin of various concentrations at 37° for 15 min and then centrifuged at 12000 rpm for 40 min. The hemolytic activity of the supernatant was measured.

Results

Taxonomic Studies of the Producing Organism

The monopodialysin-producing *Streptomyces* was isolated from a soil sample collected at Gifu prefecture. The cultural characteristics on various media of strain F4810 were observed after 10—14 days of incubation at 30°. These data are shown in Table I. The physiological properties of strain F4810 are shown in Table II. Utilization of carbon sources was examined on the Pridham and Gottlieb basal medium. *Streptomyces aureomonopodiales* is considered to be the most similar species as shown in Table II. The cultural and physiological characteristics of both strains are very similar except for the utilization of inositol. Thus *Streptomyces* sp. F4810 can be designated as *Streptomyces aureomonopodiales*.[9,10]

Production and Isolation of Monopodialysin

A typical production pattern of the hemolytic substance by *Streptomyces aureomonopodiales* is shown in Fig. 1. The maximum hemolytic activity of acetone extract is obtained at 72 hr cultivation.

7) A. Raz, A. Schurr, A. Livne, and R. Goldman, *Biochem. Pharmacol.*, **22**, 3129 (1973).
8) J.T. Dodge, C. Michell, and D.J. Hanahan, *Arch. Biochem. Biophys.*, **100**, 119 (1963).
9) S.A. Waksman, "The Actinomycetes," Vol. 2, The Williams and Wilkins Co., Baltimore, 1961.
10) E.B. Shirling and D. Gottlieb, *International Journal of Systemic Bacteriology*, **18**, 69, 279 (1968); *idem, ibid.*, **19**, 391 (1969); *idem, ibid.*, **22**, 265 (1972).

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Table I. Cultural Characteristics of *Streptomyces* sp. F 4810

	Growth	Aerial mycelium	Soluble pigment
Glucose asparagine agar	White Powdery	Reddish brown Small colony	Light brown
Glycerol asparagine agar	White Small cottony	Dark brown Small colony	Brown
Starch agar	White Small cottony	Brown Colony	None
Tyrosine agar	White Powdery, cottony	Dark brown Colony	Brown
Nutrient agar	Colorless	White to brownish grey Small colony	Brown
Yeast maltose agar	White Cottony	Brown Colony	Brown
Oatmeal agar	White Powdery Small cottony	Brown to brownish grey Small cottony	White brown
Glucose peptone agar	White Powdery	Brown to black	Dark brown
Milk	Colorless	Dark brown, Ring	Brown
Peptone yeast iron agar	Colorless	Black Small colony	Dark brown

Table II. Comparison of *Streptomyces* sp. F 4810 with *Streptomyces aureomonopodiales*

	St. sp. F 4810	*St. aureomonopodiales*
Spore chain morphology	RF[a]	RF[a]
Spore surface	Smooth	Smooth
Aerial mass color	White	White
Reverse side of colony	Dark brown	Not dist.
Soluble pigment	Melanoid (+) Yellow to brown	Melanoid (+) Yellow
Gelatin liquefaction	−	−
Coagulation and peptonization of milk	−	−
Hydrolysis of starch	+	+
Utilization of carbohydrate		
arabinose	+	+
xylose	+	+
glucose	+	+
fructose	+	+
rhamnose	−	−
sucrose	−	−
raffinose	+	+
mannose	+	+
inositol	−	+

a) Rectiflexiblis.

After 72 hr incubation at 30° the mycelium was separated from the culture broth by centrifugation. A schematic representation of the isolation procedure from the mycelium is shown in Chart 1.

The cytolytic principle was extracted with three times 2.1 l of acetone from the 700 g of wet mycelium. The acetonic extract was concentrated *in vacuo* and suspended in distilled water. The cytolytic substance in the water was extracted three times with ethyl acetate

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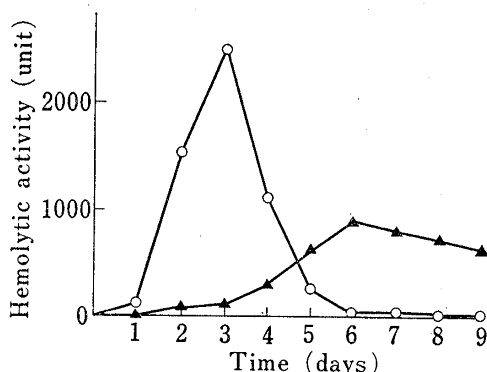

Fig. 1. Production of Hemolytic Principle by *Streptomyces aureomonopodiales*

○—○, acetone extract from mycelium,
▲—▲, culture filtrate.

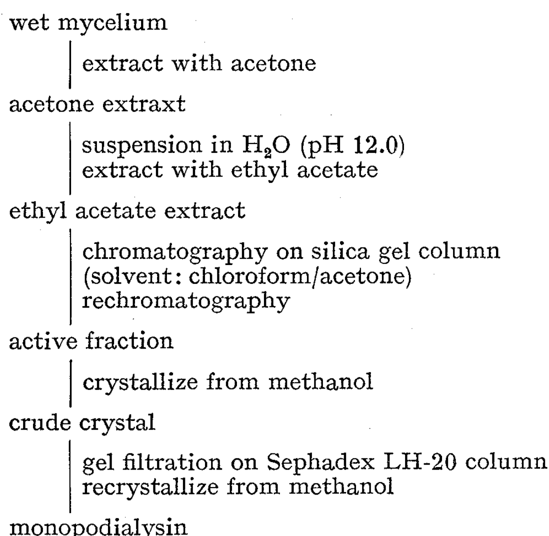

Chart 1. Isolation of Monopodialysin from Mycelium of *Streptomyces aureomonopodiales*

at pH 12.0. The extract was concentrated to dryness, yielding 10 g of the residue. The residue was dissolved in a small amount of chloroform, placed on a column of silica gel (Wakogel C-100, 120 g) and developed with 20, 40 and 60% (v/v) acetone/chloroform mixtures, stepwisely. The active fraction eluted by 60% acetone in chloroform was evaporated to dryness and further purified by gel filtration column of Sephadex LH-20 using methanol as solvent, followed by crystallization from methanol to yield 200 mg of colorless plate crystal

Physicochemical Properties

The molecular weight of monopodialysin was suggested to be less than 1000 by gel filtration (Sephadex LH-20), since mass spectroscopy analysis performed did not give satisfactory results. Monopodialysin is very soluble in pyridine, dimethyl sulfoxide (DMSO), slightly soluble in methanol, acetone and ethyl acetate, sparingly soluble ether, chloroform and benzene, and insoluble in water. The elemental analysis gave: C, 62.20%, H, 9.55%; d.p. above 179°, $[\alpha]_D^{25}$ +8° (c=0.5, ethanol). The UV spectrum of monopodialysin is shown in Fig. 2. Its infrared spectrum shows characteristic absorption at 3350, 1730 and 1630^{-cm}

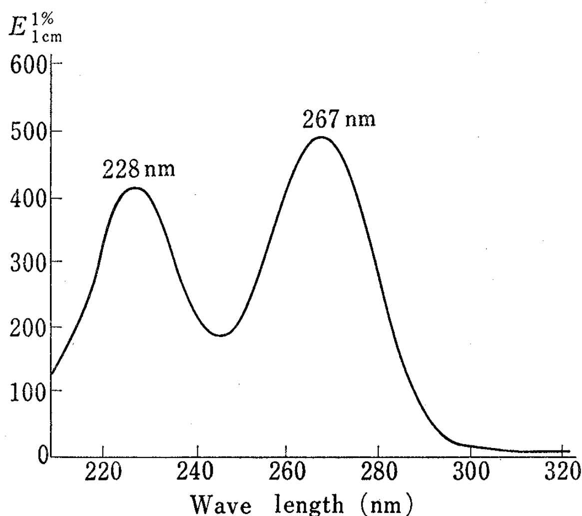

Fig. 2. UV Spectrum of Monopodialysin

Solvent: methanol.

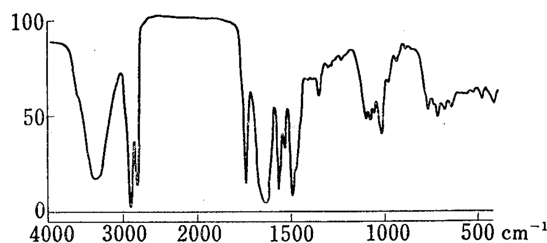

Fig. 3. Infrared Spectrum of Monopodialysin (KBr)

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as shown in Fig. 3. Color reaction: positive, Dittmer-Lester;[11] negative, $FeCl_3$ and Molish. Monopodialysin is stable in organic solvents for a few months at room temperature. Most of the cytolytic activity of monopodialysin is lost rapidly in the present of sodium chloride, especially Cl^-, on standing at 37° for 10 min but stable in sodium phosphate buffer. Therefore experiments were carried out in isotonic phosphate buffer (pH 7.4).

Biological Properties

i) Hemolytic Activity——As illustrated in Fig. 4 monopodialysin at the concentration of 2.0 μg/ml exhibits hemolysis on sheep erythrocyte. Erythrocyte of various mammalian species are found to be not equivalent in their susceptibility to destruction by monopodialysin as shown in Table III. When 5.5 μg/ml of monopodialysin was preincubated at 37° for 15 min with soy bean lecithin, cholesterol and sucrose in various levels, the remaining hemolytic activity in the reaction mixture was estimated. Addition of cholesterol (10^{-2} μmol/ml) or lecithin (10^{-1} μmol/ml) can completely prevent lysis of erythrocytes as seen in Fig. 5, but sucrose, nonpermeable solute, exhibited no inhibitory effect even at high concentration.

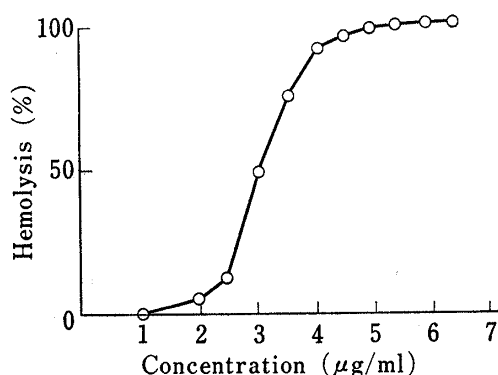

Fig. 4. Hemolytic Activity of Monopodialysin on Sheep Erythrocyte

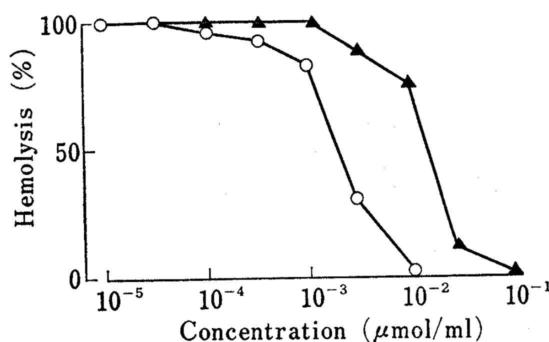

Fig. 5. Inhibitory Effect of Lecithin or Cholesterol on Hemolytic Activity of Monopodialysin

▲—▲, lecithin,
○—○, cholesterol,
Monopodialysin: 5.5 μg/ml.

TABLE III. Sensitivity on Various Mammalian Erythrocyte to Monopodialysin

Source of erythrocyte	Relative sensitivity[a] (μg/ml)
Bovine	1.4
Sheep	2.0
Guinea pig	2.3
Rabbit	2.5
Mouse	2.7
Rat	3.6

a) Minimum essential concentration to cause hemolysis.

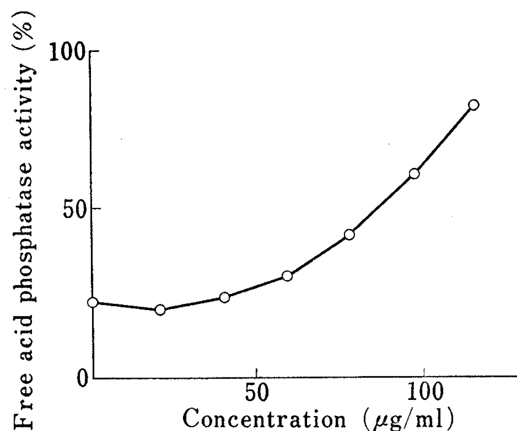

Fig. 6. Effect of Monopodialysin on the Release of Acid Phosphatase from Rat Liver Lysosomes

ii) Binding to Bovine Erythrocyte Ghost——The hemolytic activity was not found in the supernatant centrifuged after incubating monopodialysin with bovine red blood cells. The result indicates that most of monopodialysin was adsorbed to cell membrane.

11) J.C. Dittmer and R.L. Lester, *J. Lipid Res.*, **5**, 126 (1964).

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iii) Effect on Lysosome——From Fig. 6 cytolytic effect on lysosomal membrane from rat liver was shown at concentration of monopodialysin higher than 50 μg/ml.

iv) Effect on Bacterial Protoplast——Further insight into the mechanism of action of monopodialysin on membranes came from the experiment on protoplast of *Bacillus subtilis*. The protoplasts were lysed completely by this cytolytic substance at 100 μg/ml concentration.

v) Antimicrobial Activity——The antimicrobial activity of monopodialysin was determined by the paper disc method, but monopodialysin at 1.0 mg/disc (solvent: DMSO) demonstrated no inhibition of gram-positive, gram-negative bacteria and some fungi (*i.e.*, *Streptococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Penicillium chrysogenum*, *Aspergillus niger* and *Candida albicans*). The explanation of the insensitivity of whole bacteria in contrast to wall-less bacteria is that the unaltered bacterial cell wall physically prevented access of this lysin to the underlining membrane which is the site of action of monopodialysin.

vi) Toxicity——Intraperitoneal administration of monopodialysin suspended in saline containing 5% acacia at a dose of 400 mg/kg showed no toxic symptom to mice within 48 hr.

Discussion

Only a few hemolytic substances have been reported to be produced by *Streptomyces*, *e.g.* siolipin[12] formed by *Streptomyces sioyaensis* and several polyene antibiotics. Monopodialysin can be differentiated from siolipin by its chemical properties. Monopodialysin consisting of C,H, and O has the molecular weight of less than 1000, C=C bonds, carboxyl C=O, OH functions and a number of $-CH_2-$ groups. Its hemolytic action is inhibited by cholesterol in a similar fashion to that of polyene antibiotics.[13] The fact that acetylated monopodialysin indicated the same hemolytic potency as did monopodialysin, suggested that OH functions are not necessary for the hemolytic activity.

The binding of monopodialysin to bovine erythrocyte ghosts and the antagonism by cholesterol or lecithin suport the interaction of between monopodialysin and cholesterol or phospholipids in the erythrocyte membrane which alters the structure of the membrane to cause hemolysis.

Monopodialysin shows the cytolytic action on protoplast of *Bacillus subtilis* but no detectable antibacterial activity. The preferential sensitivity suggests that cell wall of bacteria can act as an effective barrier or monopodialysin is inactivated before reaching the cell membrane.

The chemical structure of monopodialysin and its interaction against cholesterol will be reported in following papers.

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