A NEW 5′-NUCLEOTIDASE INHIBITOR, NUCLEOTICIDIN

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL PROPERTIES

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A new 5′-nucleotidase inhibitor named nucleoticidin was produced by *Pseudomonas* sp. YM-3229G. It was isolated from a fermentation broth by trichloroacetic acid extraction, ethanol precipitation and Dowex 1 and DEAE-52 column chromatography. It inhibited 5′-nucleotidase activity of snake venom and rat liver membrane. It also showed antitumor activity against solid type Sarcoma 180.

In the course of screening for new enzyme inhibitors, we found a new compound named nucleoticidin inhibiting 5′-nucleotidase activity present in snake venom and rat liver membrane. It showed antitumor activity. This paper deals with the taxonomy of the producing strain *Pseudomonas* sp. YM-3229G, the isolation of the active principle and some biological properties of nucleoticidin.

Assay Method of 5′-Nucleotidase Inhibitor

The reaction mixture (total volume of 0.5 ml) consisted of 55 mM Tris-HCl (pH 8.5), 5.5 mM $MgCl_2$, 1.1 mM AMP and 10 mM sodium potassium tartrate and an appropriate amount of 5′-nucleotidase and the inhibitor. After incubation at 30°C for 30 minutes, the enzyme reaction was stopped by addition of 0.5 ml of 10% trichloroacetic acid. The amount of inorganic phosphate liberated from AMP by the enzymatic reaction was colorimetrically measured by the FISKE-SUBBAROW method[1,2] as follows: to the reaction mixture was added 50 μl of 1% Triton X-100, 3.5 ml of water and 0.5 ml of coloring reagent (2.5% w/v ammonium molybdate in 5 N H_2SO_4) and the reaction mixture was left for 20 minutes at room temperature. The liberated phosphate was determined by measuring the absorbance at 660 nm. A unit was defined as the reciprocal of the concentration, expressed as mg/ml, which showed 50% inhibitory activity. That is, one unit means that a solution of the sample showed 50% inhibitory activity at 1 mg/ml.

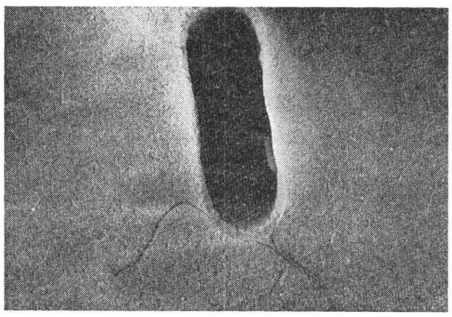
Taxonomic Studies

The microorganism used for the isolation of nucleoticidin was isolated from a soil sample collected in Maruyama Park in Sapporo City, and had following properties.

Morphology

On nutrient agar medium it grows in the form of rod with a size of 0.6 to 0.8×1.5 to 3.0 μm; it is motile with polar flagella. It is non-sporulating and Gram-negative and does not show pleomor-

Fig. 1. Electron micrograph of *Pseudomonas* sp. YM-3229G.
Bar represents 1 μm.



phism.

Cultural and Physiological Characteristics

The cultural and physiological characteristics of strain YM-3229G are summarized in Tables 1 and 2, respectively. D-Glucose, starch or glycerol are used as a sole source of carbon, but the following compounds could not be used: L-arabinose, D-xylose, D-fructose, sucrose, inositol, L-rhamnose, raffinose, D-mannitol, D-galactose, maltose, trehalose, lactose, D-sorbitol and esculin.

Based on the properties described above, strain YM-3229G was similar to *Pseudomonas alcaligenes*[3]. However, in contrast to *P. alcaligenes*, this strain produced mucous substance in media containing starch and did not grow at 41°C. From these results, it was concluded that the strain YM-3229G was not identical with *P. alcaligenes*.

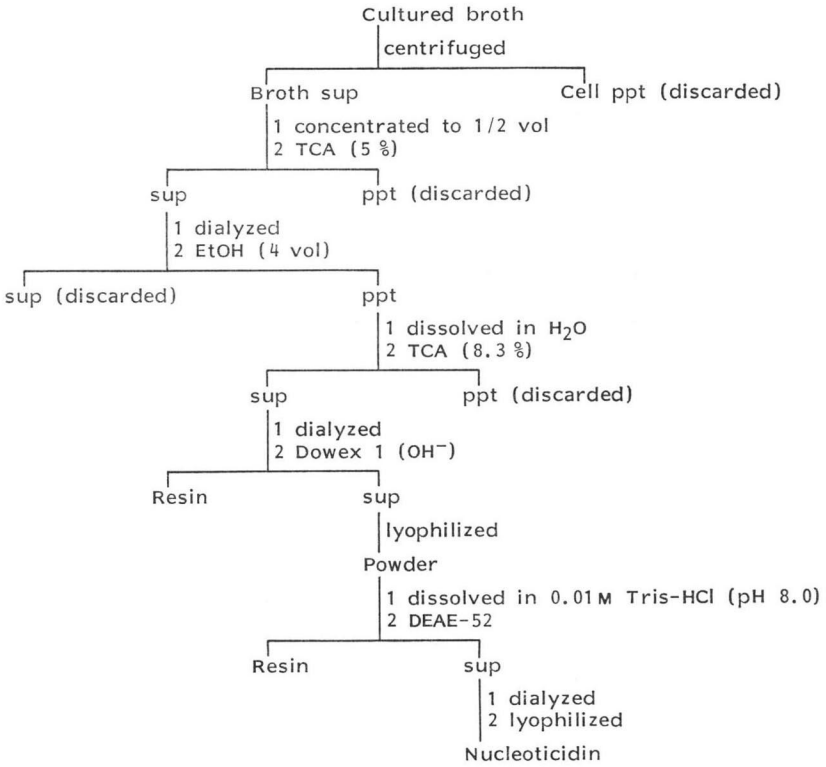
Table 1. Cultural characteristics.

Culture condition	Characteristics
Nutrient agar, 28°C, 2~6 days	Good growth, whitish creamy in color.
Nutrient broth, 28°C, 2~6 days	Lightly viscous pellicle on surface. Culture becomes turbid throughout.
Litmus milk, 28°C, 2~6 days	Peptonized; pH turns slightly alkaline.
Gelatin stab	Liquefied.

Table 2. Physiological characteristics.

	Response
Hydrolysis of starch	−
Liquefaction of gelatin	+
Hydrolysis of casein	−
Catalase reaction	−
Indole formation	−
Hydrogen sulfide formation	−
Urease reaction	−
Reduction of nitrate	+
Utilization of citrate (Simmons medium)	+
Egg yolk reaction	−
Oxidase reaction (Kovac's test)	+
Voges-Proskauer reaction	−
MR test	+
Requirement of growth factors	None
Growth in anaerobic condition	−
Utilization of inorganic nitrogen source	
Ammonium nitrogen	+
Nitrate nitrogen	Weak
Production of soluble pigment	−
Accumulation of poly-β-hydroxybutyrate	−
Denitrification	−
O-F test	Aerobic: Neither acid nor gas from glucose
Growth temperature	Moderate to abundant growth at 15~37°C, but no growth at 40°C.

Fig. 2. Purification of nucleoticidin.



The electron microscopic picture of the strain is shown in Fig. 1.

Fermentation

A loopful of cells of strain *Pseudomonas* sp. YM-3229G on an agar slant was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a medium composed of glucose 2.5%, soybean meal 1.5%, cotton seed meal 0.5%, meat extract 1.0%, $CaCO_3$ 0.3% and NaCl 0.2%. The pH of the medium was adjusted to 7.0 before sterilization. The flasks were incubated on a rotary shaker at 27°C for 48 hours. A 2-ml aliquot of the culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the same medium and fermentation was continued at 27°C for 72 hours with shaking, At the harvest time, pH was 8.0 and potency was 2.4×10^5 units/1,900 ml (126 units/ml).

Isolation

The purification procedure of nucleoticidin is outlined in Fig. 2 and Table 3. After fermentation, the cultured broth was centrifuged to remove the cells. The supernatant was concentrated to one-half of the original volume by rotary evaporator below 50°C. In order to remove proteins, trichloroacetic acid was added to the supernatant solution. After centrifugation, the supernatant was made neutral, dialyzed against water and then 4 volumes of ethanol were added. The precipitate obtained by centrifugation was dissolved in water and solid trichloroacetic acid was added to a final concentration of 8.3%. After centrifugation, the supernatant was made neutral, dialyzed against water, and Dowex 1 (OH^-) was added. The mixture was centrifuged and the supernatant was lyophilized. The crude powder was dissolved in 0.01 M Tris-HCl (pH 8.0) and DEAE-52, equilibrated

Table 3. Purification of nucleoticidin.

Purification step	Total weight (mg)	Total activity ($\times 10^3$ units)	Specific activity (units/mg)	Yield (%)
Broth supernatant	1,900 ml (25.3 mg/ml)	240	5	
Ethanol	13,775	207	15	86
	5,000	75		
Dowex 1 (OH^-)	3,028*	48	16	55
	1,400	22		
DEAE-52	1,056**	26	25	55

* 3,028 mg came from 5,000 mg present in EtOH fraction.
** 1,056 mg came from 1,400 mg in Dowex 1 (OH^-) fraction.

Table 4. The effect of nucleoticidin on various 5′-nucleotidase.

Source	IC_{50} (μg/ml)
Snake	40
Rat liver	48

with the same buffer, was added. The mixture was centrifuged and the supernatant was dialyzed against water and then lyophilized to give nucleoticidin as a colorless powder (25 units/mg).

Biological Properties

Nucleoticidin inhibited 5′-nucleotidase activity from snake venom (*Crotalus atrox*, Sigma) and that from rat liver membrane obtained by the procedure of NAKAMURA[4] (Table 4). Nucleoticidin also reduced the growth of Sarcoma 180 solid tumor in rat. As a polysaccharide, it is supposed to act on the growth of tumor through the immunological system. The details of the biological studies will be published elsewhere. Among nearly 50 known compounds tested for inhibitory activity of 5′-nucleotidase at 20 μg/ml, such as benzylpenicillin, streptomycin, erythromycin, mitomycin C, tetracycline, rifampicin, daunorubicin, bleomycin, viomycin, actinomycin D, neocarzinostatin, elastatinal, antipain, chymostatin, caffeine, theophylline, lentinan, concanavalin A, picibanil and heparin, only concanavalin A inhibited nucleotidase activity from rat liver membrane. In the case of the snake venom enzyme, no compound showed inhibitory activity at 20 μg/ml. Therefore, nucleoticidin is a useful reagent for 5′-nucleotidase study as well as for study of immunological system. In addition, it has potential antitumor activity.

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