Screening and Identification of the Phospholipase D-producing *Streptomyces*[†]

Tsutomu YAMAGUCHI, Yoshio OKAWA, Kengo SAKAGUCHI and Naoki MUTO

Research Laboratory, Toyo Jozo Co., Ltd., Ohito-cho, Shizuoka-ken

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About 2,000 strains of streptomycetes and 200 strains of molds were tested for egg yolk-degrading activity. A strain isolated from soil was found to produce the strong activity in the culture medium.

The egg yolk-degrading substance was found to be phospholipase D and the producing strain was identified as *Streptomyces hachijoensis*. It was found out that the strains producing egg yolk-degrading substance appeared with high frequency in whirl-forming species in genus *Streptomyces*.

There have been a number of reports on phospholipase D of plant materials such as carrot and cabbage.¹⁻³⁾ It has long been thought that phospholipase D is virtually absent in microorganisms. Recently, however, two bacteria were found to produce the enzyme, that is, *Haemophilus parainfluenzae*⁴⁾ and *Corynebacterium ovis*.⁵⁾

In order to obtain microorganisms which are able to produce phospholipases, about two thousand strains of streptomycetes and two hundred strains of molds were examined for the producibility of those enzymes by using egg yolk agar plate for the enzyme assay. A strain isolated from soil excreted a large amount of the egg yolk-degrading substance. Thin-layer chromatographic analysis revealed that the substance produced phosphatidic acid and choline from lecithin.

The present paper deals with the screening for the production of egg yolk-degrading substance, identification of the substance, phospholipase D, and taxonomical identification of a potent culture belonging to *Streptomyces*. The relationship between morphological characteristics and producibilities of the egg yolk-degrading substance in *Streptomyces* are also described.

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MATERIALS AND METHODS

Assay of the egg yolk-degrading activity. Egg yolk plates were prepared as follows:

Beat yolk of four eggs (free from white) was suspended in 100 ml of saline and 25 g of celite were added to the suspension. The mixture was then filtered with Toyo-filter paper No. 2. One part of the filtrate was added to 9 parts of melted 2% agar solution (at about 60°C). To prevent infection by microorganisms, streptomycin was added to the mixture at the final concentration of 10 µg/ml. Ten milliliter portion of the egg yolk agar was poured into a petri-dish.

Sample to be tested was absorbed to a pulp disc (diameter: 8 mm) which was dried on filter paper at room temperature for about 30 min.

After the disc was put on the egg yolk plate, the plate was incubated at 37°C for 15 hr and the diameter of the clear zone (in mm) formed around the pulp disc was measured.

Culture. About 2,000 cultures of streptomycetes and 200 cultures of molds were isolated from soils and plants and subjected to examination of the producibilities of egg yolk-degrading substance.

In the case of streptomycetes, a 100 ml aliquot of medium in a 500 ml-Erlenmeyer flask sterilized at 120°C for 20 min was inoculated with a strain and cultivated at 30°C for 4 or 5 days with shaking.

The cultured broth was centrifuged to obtain clear supernatant which was used for assay of the activity. The medium used consisted of polypeptone, 0.75%; meat extract, 0.75%; soluble starch, 1%; NaCl, 0.3% and MgSO₄·7H₂O, 0.1%, pH 7.2.

In screening tests for molds, the medium consisted of potato extract, 10%; C.S.L., 0.5%; KH₂PO₄, 0.5%; MgSO₄·7H₂O, 0.25%; pH 6.5.

Thin-layer chromatography. Thin-layer chromatography (TLC) was carried out on silica gel G with chloroform-methanol-water (65:25:3, by volume) as the developing solvent. The substances on the TLC were detected by spraying ninhydrin solution or 50% H_2SO_4 (v/v). R_f values of authentic phosphatidylethanolamine, phosphatidic acid and ethanolamine were 0.66, 0.91 and 0.11, respectively.

Enzymes. Phospholipase D from cabbage was purchased from BDH Chemicals Ltd. (Poole, England) and the others from Seikagaku Kogyo Co., Ltd. (Tokyo).

Chemicals. Phosphatidylethanolamine and lecithin were prepared from egg yolk according to the method of Dawson.⁶⁾ Phosphatidic acid was prepared according to the method of Kates⁷⁾ by hydrolysis of lecithin with phospholipase D of cabbage.

Ethanolamine hydrochloride was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). Other chemicals were of the purest grade commercially available.

RESULTS

1. Screening of strains capable of degrading egg yolk

Among about 2,000 cultures of *Streptomyces* examined, 4 cultures were found to produce the egg yolk-degrading substance (Table I).

TABLE I. EGG YOLK-DEGRADING ACTIVITIES IN SIX CULTURES OF STREPTOMYCETES AND MOLDS

Microorganism	Strain No.	Activity (mm ϕ)	
		3 day	5 day
<i>Streptomyces</i> sp.	A 853	12.8	14.0
"	A 1025	14.3	15.6
"	A 1143	18.2	19.6
"	A 1579	16.0	16.8
Mold			
unidentified	M 53	10.8 ^{a)}	11.7 ^{a)}
"	M 76	10.0 ^{a)}	10.5 ^{a)}

^{a)} Cloudy zone

In this Table, *Streptomyces* sp. A 1143 showed the highest potency of the egg yolk-degrading activity. This strain was isolated from soil collected at Ikeda city, Osaka prefecture.

In the molds, 2 cultures among about 200 cultures showed the egg yolk-degrading activity. However, the activities of both cultures

were low. Also some other cultures of molds gave small cloudy zones on the egg yolk plate.

2. Identification of egg yolk-degrading substance

These selected microorganisms have a possibility to produce simultaneously different enzymes, because an egg yolk suspension is claimed to be cleared by protease of *Streptomyces* (Pronase), phospholipase of snake venom, lipase of *Candida*, pancreatic lipase and lipoprotein lipase.

In our preliminary experiments, pancreatic lipase, phospholipase A of snake venom, trypsin and some detergents such as SDS, deoxycholate and Triton X-100 made clear zone on the egg yolk plate. But pronase, phospholipase C of *Clostridium*, lipases of *Candida* and *Rhizopus* showed no appreciable reaction on the plate.

Since *Streptomyces* sp. A 1143 gave the highest enzymatic activity among the selected cultures, it was used in the following investi-

FIG. 1. Thin-layer Chromatography of the Reaction Products of Phosphatidylethanolamine with the Egg Yolk-degrading Substance Produced by *Streptomyces* sp. A 1143.

Reaction: Broth filtrate (0.5 ml) of *Streptomyces* sp. A 1143 was added to the mixture of 40 mM phosphatidylethanolamine (0.2 ml) and 0.05 M Tris-HCl buffer (pH 7.5, 0.3 ml). The mixture was incubated at 37°C for 20 min.

a: Standard phosphatidylethanolamine, b: Reaction products, c: Standard ethanolamine, d: Standard phosphatidic acid.

Solvent system: chloroform-methanol-water (65:25:3), Plate: silica gel G, Detecting reagent; 50% sulfuric acid.

gations for elucidation of the egg yolk-degrading substance.

The broth filtrate of *Streptomyces* sp. A 1143 was added to the suspension of phosphatidylethanolamine and the mixture was incubated at 37°C for 20 min. The reaction products were analysed by thin-layer chromatography.

As shown in Fig. 1, two substances having *R_f* values of 0.11 and 0.91 were newly formed from phosphatidylethanolamine (*R_f*: 0.66).

On thin-layer chromatogram, these two substances corresponded to ethanolamine and phosphatidic acid.

The egg yolk-degrading substance can also attack lecithin to form phosphatidic acid and choline. From these experiments, the main substance giving egg yolk-degrading activity was considered to be phospholipase D.

3. Effect of nutrient sources on the enzyme production

1) Effect of carbon sources. The effect

of several carbon sources on production of the egg yolk-degrading substance was examined.

None of the carbon sources employed stimulated the enzyme production. Glucose, sucrose, maltose and fructose were not suitable for the enzyme production.

2) *Effect of nitrogen sources.* Table II shows the effect of several organic and inorganic nitrogen sources on production of the egg yolk-degrading substance.

Meat extract, C.S.L. and yeast extract were the most suitable sources for the enzyme production.

3) *Effect of phospholipid.* Since many microbial enzymes have been reported to be formed inducibly, the effect of phospholipid on the above enzyme production was examined.

The results shown in Fig. 2 indicate that lecithin did not show any significant effect on the production of egg yolk-degrading substance.

TABLE II. EFFECT OF NITROGEN SOURCES ON PRODUCTION OF EGG YOLK-DEGRADING SUBSTANCE PRODUCED BY *Streptomyces* sp. A 1143

Nitrogen source was added to a medium containing 1% soluble starch, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3% KH_2PO_4 and 0.7% $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$. The strain was inoculated in 100 ml medium in a 500 ml-Erlenmeyer flask and incubated at 30°C on rotary shaker.

N. source	Conc. (%)	Activity (mm ϕ)		Growth
		3 day	5 day	
Polypeptone	1, 0	8.4	8.5	+++
	0, 5	8.4	8.5	+++
Casamino acid	1, 0	—	—	++
	0, 5	—	—	++
Yeast ext.	1, 0	13.2	14.3	+++
	0, 5	14.3	15.6	+++
Meat ext.	1, 0	18.8	20.5	+++
	0, 5	18.2	19.4	+++
Soy bean meal	1, 0	12.4	13.3	+++
	0, 5	10.8	11.7	++
CSL	1, 0	16.0	16.8	+++
	0, 5	17.1	17.8	+++
Asparagine	1, 0	—	—	++
	0, 5	—	—	+
Urea	0, 5	—	—	+
	0, 3	—	—	+
$(\text{NH}_4)_2\text{SO}_4$	0, 5	—	—	+
	0, 3	—	—	+
NaNO_3	0, 5	—	—	+
	0, 3	—	—	+

FIG. 2. Effect of Phospholipid on the Production of Egg Yolk-degrading Substance by *Streptomyces* sp. A 1143.

Lecithin (1%) was added to the following media. A: soluble starch, 1.0%; meat extract, 0.75%; peptone, 0.75%; NaCl, 0.3%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1%; pH 7.2, B: soluble starch, 1.0%; soy bean meal, 2.0%; NaCl, 0.3%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1%; pH 7.2, C: soluble starch, 2.0%; soy bean meal, 0.2%; NaCl, 0.25%; yeast extract, 0.5%; CaCO_3 , 0.35%.

Cultural conditions were the same as those in Table II.

—, Control; ----, Control + lecithin (1%).

4. Taxonomical studies of *Streptomyces* sp. A 1143

Morphological characteristics. Many synthetic and natural media were used for morphological characterization of the strain. Ammonium-Czapek agar which gave good morphological development was used for the microscopic observations.

The morphological characteristics are summarized as follows; (1) Aerial mycelium: Straight to long and bent, primary and secondary whirls, no spirals; $0.8 \sim 1.0 \mu$ in width. (Fig. 3). (2) Conidium: Oval to short cylindrical; $0.6 \sim 0.8 \times 1.3 \sim 1.5 \mu$ in size; smooth surface under electron microscopy. (Fig. 4). (3) Substrate mycelium: Flexuous to wavy, many branches; $0.6 \sim 0.7 \mu$ in width; no fragmentation and segmentation.

Cultural and physiological characteristics.

The cultural and physiological characteristics of *Streptomyces* sp. A 1143 are shown in Tables III and IV.

FIG. 3. Aerial Mycelia of *Streptomyces* sp. A 1143 (photomicrograph).

FIG. 4. Conidia of *Streptomyces* sp. A 1143 (electron micrograph).

From the characters described in these Tables, the strain A 1143 certainly belongs to genus *Streptomyces* and is assigned to the section *Verticillati* in "the International Streptomyces Project (ISP) definition."⁸⁻¹⁰⁾

These characters of the strain have some resemblances to those of *Streptomyces griseoverticillatus* Shinobu *et al.*,¹¹⁾ *Streptomyces cinnamomeus* Benedict *et al.*,^{12,13)} and *Streptomyces hachijoensis* Yamaguchi.¹⁴⁾ By comparison of the taxonomical properties of the strain A 1143 with those of the type cultures of *S. griseoverticillatus* OEU 722, *S. cinnamomeus* IFO 12852 and *S. hachijoensis* IFO 12782, it became clear that the strain A 1143 most related to *S. hachijoensis*. Since the strain A 1143 is not clearly differentiated as another species from *Streptomyces hachijoensis*, the authors conclude that the strain A 1143 belongs to *Streptomyces hachijoensis*.

TABLE III. CULTURAL CHARACTERISTICS OF *Streptomyces* sp. A 1143^{a)}

Medium	Growth ^{b)}	Aerial mycelium ^{c)}	Color of substrate mycelium	Soluble pigment
Czapek agar	Poor, thin	Poor; Pearl pink (3ca) to flesh pink (4 ca) to bisque (4ec)	Colorless	None
Ammonium Czapek agar	Poor, thin	Poor; Pearl pink (3ca) to flesh pink (4ca) to bisque (4ec)	Colorless	None
Glucose asparagine agar	Moderate; intruding into the medium	Moderate, Cottony; White (a) to flesh pink (4ca)	Mustard gold (2pe-2ne) to bright gold (2nc-2pc)	None
Glycerol asparagine agar (ISP No. 5)	Moderate; intruding into the medium	Poor; White (a) to light ivory (2ca)	Light wheat (2ca) to mustard gold (2ne)	None
Starch inorganic salts agar (ISP No. 4)	Moderate to poor	Poor; White (a) to pearl pink (3ca) to light ivory (2ca)	Mustard gold (2ne) to topaz (3ne)	None
Tyrosine agar (ISP No. 7)	Good; intruding into the medium	Good to excellent, Cottony, colorless droplets; Pearl pink (3ca) to light melon yellow (3ea) to white (a)	Mustard gold (2pc-2pe)	None
Nutrient agar	Moderate	Poor; White (a) to light ivory (2ca)	Mustard gold (2pe) to amber (3pe)	None
Yeast malt ext. agar (ISP No. 2)	Good; intruding into the medium	Excellent to good, Cottony, colorless droplets; Flesh pink (4ca) to bisque (4ec) to pearl pink (3ca)	Amber (3pc-3nc)	None
Oat meal agar (ISP No. 3)	Moderate; intruding into the medium	Moderate, Cottony; Pearl pink (3ca) to light melon yellow (3ea) to flesh pink (4ca)	Amber (3pc) to bright gold (2pc)	None
Glucose peptone gelatine	Good	None	Amber (3pc-3pe)	None
Skim milk	Good to excellent	None to trace; White (a)	Gold (21c) to squash yellow (2ga)	None to amber (3nc)
Peptone yeast iron agar (ISP No. 6)	Good to moderate	None to trace	Amber (3pc) to topaz (3ne)	None

a) The color terms were expressed according to "Color harmony manual," 4th ed. 1958.

b) Growth of the colony and its nature

c) Growth and nature of aerial mycelium and its color

TABLE IV. PHYSIOLOGICAL CHARACTERISTICS OF *Streptomyces* sp. A 1143

Tyrosinase reaction	Negative
Nitrite production	Positive, weak
Amylase reaction	Positive, weak
Gelatinase reaction	Negative
Reaction on milk	
Peptonization	Positive
Coagulation	Positive, weak
Cellulase reaction	Negative
Carbohydrate utilization	
Positive	D-Glucose, Trehalose, L-Inositol, D-Fructose, Sucrose
Negative	D-Galactose, L-Rhamnose, Lactose, D-Xylose, L-Arabinose, Raffinose, Mannitol

5. Producibility of egg yolk-degrading substance of whirl-forming *Streptomyces*

In the morphological studies of four strains of *Streptomyces* that were selected in the screening test (section 1), it was found out that all these strains, as well as *S. hachijoensis*, belong to whirl-forming *Streptomyces*. It was assumed that there is some relationship between whirl-forming character and the production of egg yolk-degrading substance. Therefore, the type cultures of whirl-forming *Streptomyces* were examined on their producibilities of egg yolk-degrading substance.

The results are shown in Table V.

As it was expected, eight out of ten whirl-forming species in *Streptomyces* were found to produce egg yolk-degrading substance. It

TABLE V. PRODUCIBILITIES OF EGG YOLK-
DEGRADING SUBSTANCE OF VARIOUS
WHIRL-FORMING *Streptomyces*

Medium used was polypeptone, 0.75%, meat extract, 0.75%; soluble starch, 1%; NaCl, 0.3%; MgSO₄·7H₂O, 0.1%; pH 7.2.

The strain was inoculated in 100 ml medium in a 500 ml-Erlenmeyer flask and incubated at 30°C on rotary shaker.

Streptomyces	Strain No.	Activity (mm ϕ)	
		3 day	5 day
<i>S. hachijoensis</i>	A 1143	18.2	19.6
<i>S. albireticuli</i>	IFO 12737	13.6	13.7
<i>S. flavopersicus</i>	" 12769	14.1	16.2
<i>S. griseocarneus</i>	" 12776	14.1	16.1
<i>S. mashuensis</i>	" 12888	—	9.1
<i>S. mediocidicus</i>	" 13202	—	10.0
<i>S. cinnamomeus</i>	" 12852	18.2	20.0
<i>S. roseovorticillatus</i>	OEU 462	—	—
<i>S. hirosimensis</i>	" 201	15.3	18.0
<i>S. luteovorticillatus</i>	" 486	—	—

can be said that the strains producing egg yolk-degrading substance are found with high frequency in whirl-forming *Streptomyces*.

DISCUSSION

Most reports on phospholipase D have described the enzyme from plant origin. Only a few reports have dealt with the enzyme from microorganisms. The microbial enzymes so far described were obtained from bacteria. Wen-Hsiung Ko *et al.*,¹⁵⁾ and Bättcher¹⁶⁾ demonstrated that some strains belonging to *Actinomycetes* produced lecithinase-like enzyme. However, the identification of the enzyme was not carried out. Except these report, few investigations have appeared on the formation of phospholipase D by *Actinomycetes*.

Pridham *et al.*,¹⁷⁾ reported that the genus *Streptomyces* was subdivided morphologically into seven sections which were rearranged into four sections by the International Streptomyces Project (ISP) as follows:

Rectiflexibiles which forms straight to flexuous sporophores.

Rectinaculiaperti which forms sporophores as hooks, openloop or greatly extended primitive

coils or spirals of wide diameter.

Spirales which forms sporophores as short spore chains with partial spirals; short, gnarled, or compact spirals; extended, long and open spirals.

Verticillati which forms 3 or more sporophores as verticils or whirls spaced regularly on a main hypha.

It is interesting that all of *Streptomyces* strains producing the egg yolk-degrading substance investigated here belong to the section *Verticillati*.

Further studies on this series of experiments will be reported in the near future.

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