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A New Phosphatase produced by *Streptomyces yokosukanensis*

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The cultured filtrates of Streptomyces were screened for the anti-thymidine kinase activity using the homogenate of Yoshida sarcoma cells and (methyl-^{3}H)-thymidine to obtain antitumor antibiotics. The active component produced by *Streptomyces yokosukanensis* was isolated and determined to be a new acid phosphatase (E.C. 3.1.3.2.). Production, isolation, purification, effect of pH on the activity, stability, effect of temperature on the activity, effect of inhibitors on the activity and substrate specifity are described.

The biochemical method[2] to test inhibition of RNA, DNA or protein synthesis using a homogenate of cancer cells has been reported by Nitta, *et al.* to screen antitumor antibiotics. Phenomycin[3] was the first antitumor antibiotics which had been discovered by testing inhibition of protein synthesis in cell-free system of Ehrlich carcinoma cells.

As extended the biochemical method to obtain antitumor metabolites, the cultured filtrates of 60 *Streptomyces* strains were tested for the anti-thymidine kinase activity using the homogenate of Yoshida sarcoma cells and (methyl-^{3}H)-thymidine.[4,5] Seven *Streptomyces* strains were shown to have the strong anti-thymidine kinase activity. Three strains out of the seven were observed to show the proteolytic activity[6] and the active components were determined to be proteases. The other four strains were shown to have the strong anti-thymidine kinase activity without showing the proteolytic activity and all of the active components were proven to be phosphatases.

The new phosphatase was isolated from the cultured broth of *Streptomyces* H 30-SY 3, one of the above four strains. *Streptomyces* H 30-SY 3 was isolated from a soil sample collected at Shirakiyama, Hiroshima Prefecture, and classified to belong to *Str. yokosukanensis*.[7] Production, isolation and characteristics of the phosphatase are presented in this paper.

Str. yokosukanensis was shake-cultured in shaking flasks containing a medium composed of potato starch, glucose, meat extract, peptone and various metal salts at 27—28° for 68 hr. The anti-thymidine kinase activity was measured according to Umezawa's method[5] for production and isolation of the enzyme. The active component was precipitated from the broth filtrate by saturation with ammonium sulfate. The crude powder containing the anti-thymidine kinase activity was recovered by following dialysis and reprecipitation from the retantate by addition of acetone.

The crude powder was purified further by gel filtration on a column of Sephadex G-75 and purity of the eluates containing the activity was examined by the elctrophoresis using

1) Location: *1-2-3, Kasumi, Hiroshima.*
2) K. Nitta, S. Mizuno, and H. Umezawa, *J. Antibiotics* (Tokyo), **19A**, 282 (1966).
3) S. Nakamura, T. Yajima, M. Hamada, T. Nishimura, M. Ishizuka, T. Takeuchi, N. Tanaka, and H. Umezawa, *J. Antibiotics* (Tokyo), **20A**, 210 (1967).
4) T. Hashimoto, T. Shiosaka, H. Toide, H. Okuda, and S. Fujii, *Gann*, **60**, 41 (1969).
5) Personal communication from Prof. H. Umezawa, Institute of Microbial Chemistry, Kamiosaki, Shinagawa-ku, Tokyo.
6) S. Nakamura, Y. Marumoto, H. Yamaki, T. Nishimura, N. Tanaka, M. Hamada, M. Ishizuka, T. Takeuchi, and H. Umezawa, *Chem. Pharm. Bull.* (Tokyo), **17**, 717 (1969).
7) G. Nakamura, *J. Antibiotics* (Tokyo), **16A**, 94 (1961).

0.1 M Tris-HCl buffer (pH 7.0) on a cellulose acetate film (Separax, a trade name of Jokosangyo Co.) at 1 mA/cm for 1 hr. The eluates containing the activity showed eight spots at 1.5, 3.0, 4.5, 6.5, 8.5, 9.0, 9.5, and 13.0 mm to the cathode by staining with Ponceau 3R after the electrophoresis. The active component was further purified by gel filtration on a column

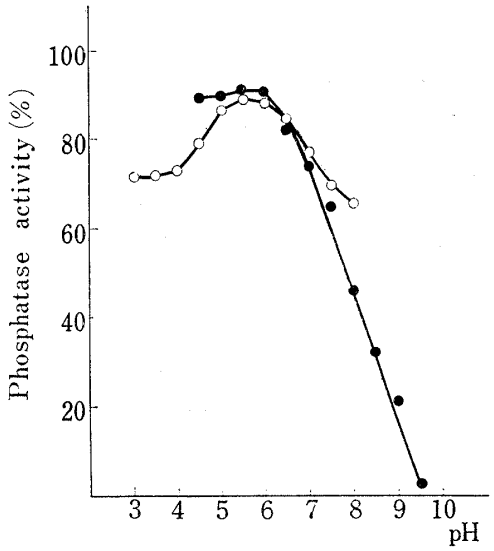

Fig. 1. Effect of pH on Phosphatase Activity

25 μl of the aqueous enzyme solution (1 μg/ml) and 25 μl of 4/10 M AcONa–AcOH buffer (pH 3.0—8.0) or 4/10 M Tris-HCl buffer (pH 4.5—9.5) was incubated with 50 μl of the (methyl-^{3}H)-thymidine-5′-monophosphate solution at 37° for 30 min.

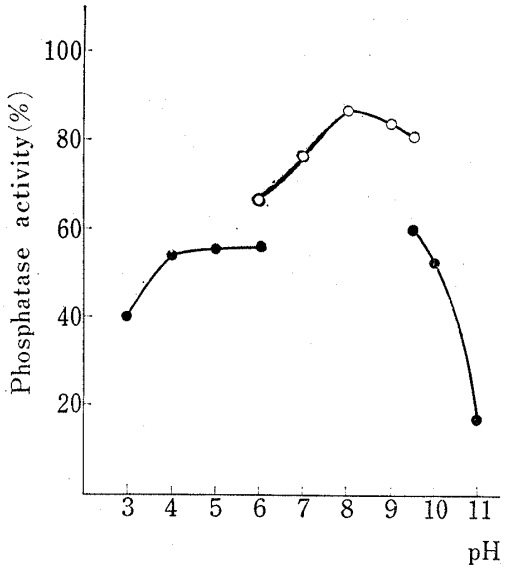

Fig. 2. pH Stability

1/10M AcONa–AcOH buffer at pH 3.0—6.0, 1/10M Tris–HCl buffer at pH 6.0—9.5 and 1/10 M borate buffer at pH 9.5—11,0 were used. 0.1 ml of the aqueous enzyme solution (2.5 μg/ml) and 0.1 ml of the buffer was kept at room temperature for 1 hr. After adjusting the pH value to 6.0 and the total volume to 1 ml using 4/10 M AcONa–AcOH buffer of various pH's, 50 μl of the resulting enzyme solution was incubated with 50 μl of the (methyl-^{3}H)-thymidine-5′-monophosphate solution at 37° for 30 min.

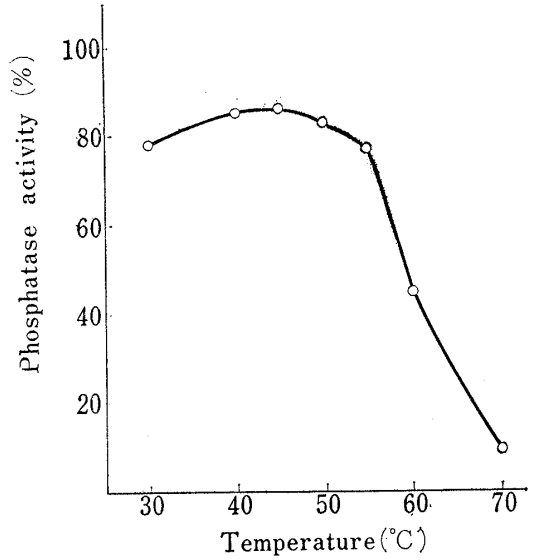

Fig. 3. Effect of Temperature on Phosphatase Activity

A mixture of 50 μl of the aqueous enzyme solution (0.5 μl/ml) and 50 μl of the (methyl-^{3}H)-thymidine-5′-monophosphate dissolved in 2/10M AcONa–AcOH buffer (pH 6.0) was incubated at the test temperatures for 30 min.

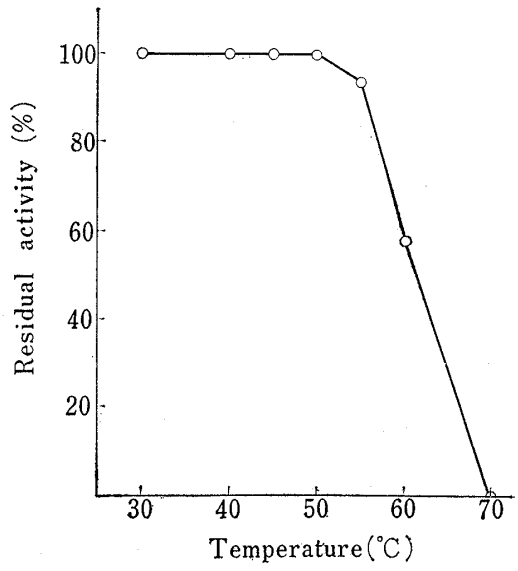

Fig. 4. Thermal Stability

50 μl of the aqueous enzyme solution (0.5 μg/ml) was heated at the test temperatures for 10 min. After cooling, the resulting solution was incubated with 50 μl of the (methyl-^{3}H)-thymidine-5′-monophosphate in 2/10M AcONa–AcOH buffer (pH 6.0) at 37° for 30 min.

of Sephadex G-200 and, thenafter, by column chromatography on DEAE-cellulose which was conducted by gradient elution of aqueous sodium chloride from 0.1 to 0.5M. The active fractions showing one spot at 4.5 mm to the cathode by the electrophoresis were finally fractionated on a column of Sephadex G-75 which afforded the most purified active component.

The active component thus obtained was considered to be a phosphatase and following enzymatic characteristics were studied using (methyl-^{3}H)-thymidine-5′-monophosphate as a substrate. The effect of pH on the phosphatase activity of the enzyme is illustrated in Fig. 1 and the optimum pH is around 5.5. The pH stability of the phosphatase at room temperature for 1 hr is shown in Fig. 2 and the enzyme is rather stable at pH 7.0—9.5, but more than half of the activity is lost below pH 3 or more than pH 11. The effect of temperature on the phosphatase activity is shown in Fig. 3. The optimum temperature is around 45°, when incubated with the substrate at pH 6.0 (1/10M AcONa–AcOH buffer) for 30 min. Nevertheless, the enzyme shows rather high phosphatase activity at 30—55°. The phosphatase is stable below 50° for 10 min in an aqueous solution, but loses about 40% of the activity at 60° for 10 min in an aqueous solution as shown in Fig. 4. Various metal ions (2.5×10^{-3}M)

Table I. Effect of Various Inhibitors on Phosphatase Activity

Chemicals	Residual activity (%)	Chemicals	Residual activity (%)
$AgNO_3$	26	L-Cysteine	83
$CaCl_2$	100	L-Cystine	45
$CoCl_2$	108	Ethylenediaminetetraacetate	90
$CuSO_4$	83	8-hydroxyquinoline	87
$FeSO_4$	57	Diisopropylfluorophosphate	0
HgCl	79	Sodium laurylsulfate	76
$HgCl_2$	0	Iodine	12
Li_2SO_4	98	Potassium permanganate	0
KCN	10	Glutathione-SH	94
$MgCl_2$	100	Glutathione-SS-G	86
$MnCl_2$	105	N-Bromosuccinimide	0
NaF	81	Cyanogen bromide	100
$NaNO_2$	103	Hydroxylamine hydrochloride	105
$ZnSO_4$	111	Ethanol	100
w-Chloroacetophenone	95	Sodium L-tartarate	100
p-Chloromercuribenzoate	34	Urethane	100
Monoiodoacetic acid	22	Potato trypsin inhibitor[a]	100

A mixture of 25 μl of the aqueous enzyme solution (1 μg/ml) and 25 μl of 1/100M inhibitor solution was kept at room temperature for 20 min. Then, the mixture was incubated with 50 μl of the (methyl-^{3}H)-thymidine-5′-monophosphate in 2/10M AcONa–AcOH buffer (pH 6.0) at 37° for 30 min.
a) 25 μl of a solution (400 μg/ml) was used.

Table II. Substrate Specificities

Disodium *p*-nitrophenylphosphate[a]	+	Guanosine monophosphate[b]	+
Lithium di-*p*-nitrophenylphosphate[a]	−	Thymidine monophosphate[b]	+
Adenosidne triphosphate[b]	−	Kanamycin-3′-phosphate[c]	+
Adenosine monophosphate[b]	+	Streptomycin-streptidine-6-phosphate[c]	+
Cytidine monophosphate[b]	+		

a) A mixture of 1 ml of the aqueous enzyme solution (1 μg/ml) and 1 ml of 1/6400M substrate in H_2O adjusted to pH 6.0 was incubated for 30 min at 37°. Optical density at 405 mμ of the mixture was determined after addition of 2 ml of 10% TCA and 4 ml of saturated Na_2CO_3 in H_2O.
b) A mixture of 1 ml of the aqueous enzyme solution (10 μg/ml) and 1 ml of the the substrate in H_2O (50 μg/ml) adjusted to pH 7.0 was incubated for 20 hr and lyophilized. The residue was chromatographed on a paper using acetone: 25% TCA=75:25. The digested substrate was detected under UV-light.
c) A mixture of 25 μl of the aqueous enzyme solution (320 μg/ml) and 1 ml of the substrate in H_2O (50 μg/ml) adjusted to pH 7.0 was incubated for 20 hr at 37°. The antimicrobial activity of the hydrolyzate for *Bac. subtilis* was determined by the cylinder agar plate method.

and enzyme inhibitors (2.5×10^{-3}M) are tested for the phosphatase activity as listed in Table I. A heavy metal ion such as silver or mercuric reduces the activity, but ethylenediaminetetraacetate does not. Further, the enzyme activity is reduced by addition of cyanate anion, diisopropylfluorophosphate or *N*-bromosuccinimide. While the enzyme activity is retained after addition of ω-chloroacetophenone, cyanogen bromide or glutathione. The substrate specifity of the enzyme (Table II) indicates that it is a phosphoric monoester hydrolase (E.C. 3.1.3.). The inactive antibiotic, kanamycin-3′-phosphate[8] or streptomycin-streptidine-6-phosphate[9] is hydrolyzed by the phosphatase to give each original active antibiotic.

The two alkaline phosphatases, H enzyme and an ordinary alkaline phosphatase, have been reported to be produced by *Str. griseus*,[9] but the phosphatase of *Str. yokosukanensis* described in this paper is an acid phosphatase (E.C. 3.1.3.2.). Thus it is a new enzyme which is different from that of *Str. griseus* origin.

Experimental

Assay Method for the Phosphatase Activity——A commercial solution (0.5 mCi/ml) of (methyl-^{3}H)-thymidine-5′-monophosphate (Schwarz Bio Research Co.,) dissolved in 50% aqueous EtOH was diluted with H_2O or a buffer to 1/200 concentration to make the standard solution of (methyl-^{3}H)-thymidine-5′-monophosphate (0.0025 mCi/ml). The reaction mixture was heated at 80° for 5 min after the enzymatic hydrolysis indicated in each figure. Fifty μl of the resulting solution (100 μl) was absorbed to DEAE-cellulose paper (Toyo Roshi Co., 1.8 cm × 1.8 cm) and the paper was washed twice with 0.001M $HCOONH_4$ for each 30 min. Then, the paper was dried at 105° for 10 min after washed with MeOH. The radio activity of the paper was measured in toluene containing DiMe-POPOP and PPO using a liquid scintillation spectrometer (Packard Co., Model 3320).

Production of the Phosphatase——The inoculation seed was shake-cultured in a 500 ml shaking flask containing 140 ml of a medium composed of 1% potato starch, 1% glucose, 0.75% meat extract, 0.75% peptone, 0.3% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$, 0.0008% $MnCl_2 \cdot 4H_2O$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0002% $ZnSO_4 \cdot 7H_2O$, and 0.0001% $FeSO_4 \cdot 5H_2O$ (pH 7.2) at 28° for 40 hr. The inoculation seed (250 ml) above cultured was used to inoculate 100 of 500 ml shaking flasks containing the same medium and cultivated at 27—28° on a reciprocal shaking machine (amplitude 7 cm, 130 rpm). The maximum anti-thymidine kinase activity (41% inhibition according to Umezawa's method[5]) was observed at 68 hr and the activity was decreased to 30% inhibition to continue the cutivation for 92 hr.

Isolation of the Crude Phosphatase——The cultured broth was harvested after 68 hr's cultivation to remove the mycelium and $(NH_4)_2SO_4$ (4.2 kg) was added to the broth filtrate (6 liters) at 0° adjusting to pH 7.0 by addition of 1N NH_4OH. The mixture was kept at 0° for 1 hr and the precipitated crude phosphatase was collected by centrifugation. The collected enzyme mixture was dialyzed in a cellophane tube against distilled water for 3 hr at 0° and 750 ml of cold acetone was added to the retantate (250 ml) to precipitate the phosphate. Thus, 12 g of the crude powder containing the anti-thymidine kinase activity was recovered by drying the precipitate *in vacuo* recovering 41% of the activity from the cultured broth.

Purification of the Phosphatase——The crude powder (12 g) dissolved in H_2O (20 ml) was fractionated to each 15 ml of fraction on a column of Sephadex G-75 (75 cm × 2 cm diameter) eluted with H_2O. The active powder (403 mg) was recovered from fractions 3—7 by lyophilization. The active powder (403 mg) dissolved in H_2O (15 ml) was further filtered on a column of Sephadex G-200 (75 cm × 2 cm diameter) to fractionate to each 15 ml of fractions. The active fractions 8—16 (135 ml) was applied to a column of DEAE-cellulose (15 cm × 1.5 cm diameter) (Cl^--type treated with 0.1M Tris-HCl buffer) (pH 7.0). The column was first eluted with 80 ml of 0.1M NaCl and then with a linear gradient of aqueous NaCl from 0.1M to 0.5M (total 800 ml). The eluate was collected in 15 ml fractions. The anti-thymidine kinase activity was detected in fractions 11—26. The active fractions were respectively dialyzed in cellophane tubes against distilled H_2O at 0° for 3 hr and lyophilized. Fractions 15—21 showed one spot at 4.5 mm to the cathode by the electrophoresis on Separax using 0.1M Tris-HCl buffer (pH 7.0) for 1 hr at 1 mA/cm and 40 V. While, fractions 11—14 showed 3 spots at 4.5, 6.0, and 9.0 mm toward the cathode and fractions 22—26 showed 2 spots at 3.0 and 4.5 mm to the same direction by the electrophoresis. The active powder (76.2 mg) from fractions 15—21 dissolved in H_2O (5 ml) was purified on a column of Sephadex G-75 (95 cm × 1.5 cm diameter) eluted with H_2O and the eluate was collected in each 5 ml fraction. The most purified phosphatase showing

8) S. Kondo, M. Okanishi, R. Utahara, K. Maeda, and H. Umezawa, *J. Antibiotics* (Tokyo), **21A**, 22 (1968).
9) *a*) R. Nomi and O. Nimi, *Agr. Biol. Chem.* (Tokyo), **33**, 1459 (1969); *b*) O. Nimi, H. Kyohara, T. Mizoguchi, Y. Ohata, and R. Nomi, *Agr. Biol. Chem.* (Tokyo), **34**, 1150 (1970).

one spot at 4.5 mm toward the cathode by the electrophoresis (7 mg) was obtained from fractions 9—12 recovering 0.8% of the anti-thymidine kinase activity from the cultured broth.

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