DEPHOSTATIN, A NOVEL PROTEIN TYROSINE PHOSPHATASE INHIBITOR PRODUCED BY *Streptomyces*

I. TAXONOMY, ISOLATION, AND CHARACTERIZATION

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A novel inhibitor of protein tyrosine phosphatase, dephostatin, was isolated from the culture broth of a strain of *Streptomyces*. The active principle was extracted from the broth filtrate with ethyl acetate and purified by silica gel chromatography and by HPLC. Dephostatin inhibited protein tyrosine phosphatase prepared from a human neoplastic T-cell line with an IC_{50} at 7.7 μM. The inhibitory pattern of dephostatin was competitive against the substrate. Dephostatin inhibited the growth of Jurkat cells.

Protein tyrosine phosphatase (PTPase) is considered to regulate the intracellular signal transduction connected with tyrosine kinase. Tyrosine kinases associated with the product of *src*-family protooncogenes are inactivated by phosphorylation of a tyrosine residue near their carboxyl terminus[1]. Dephosphorylation of this site by PTPase causes activation of these tyrosine kinases[2]. In fact, recent findings indicate that a transmembrane PTPase, CD45[3,4], is required for induction of early tyrosine phosphorylation of intracellular proteins by $p59^{fyn}$ or $p56^{lck}$ in T-cells for T-cell activation[5,6]. On the other hand, a protein-serine/threonine kinase, $p34^{cdc2}$, which controls transition from the G_2 phase of the cell cycle into mitosis, is activated by the CDC25-catalyzed dephosphorylation of the tyrosine residue in its ATP-binding site[7].

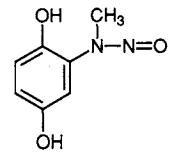
Therefore, we have screened for inhibitors of PTPase from microbial secondary metabolites to study the significance of PTPase on signal transduction and to discover immunosuppressant or anticancer drugs. As a result, we found a novel compound from the culture broth of *Streptomyces* sp. MJ742-NF5 and named it dephostatin (Fig. 1). In this report, we describe the taxonomy and fermentation of the producer strain, and the isolation and biological properties of dephostatin. The structure elucidation studies by spectral analyses and chemical derivation will be reported separately[8].

Materials and Methods

Materials

Human acute T-cell leukemia cell line Jurkat (clone E6-1) was obtained from Ms. M. OHSONO, Institute for Chemotherapy, Numazu. *o*-Phospho-L-tyrosine was purchased from Sigma, and malachite green oxalate, from Kanto Chemical Co., Inc.

Fig. 1. Structure of dephostatin.



Microorganism

Strain MJ742-NF5 was isolated from a soil sample collected in Numazu, Shizuoka prefecture, Japan, and has been deposited in the National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan, under the accession number FERM P-13223.

Isolation of Dephostatin

Spores of strain MJ742-NF5 were inoculated into several 500-ml Erlenmeyer flasks, each containing 100 ml of a medium composed of 2.0% glycerol, 2.0% dextrin, 1.0% Soypeptone, 0.3% yeast extract, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$ and cultured at 27°C for 2 days on a rotary shaker. Three ml of the seed culture was inoculated into each of one hundred 500-ml Erlenmeyer flasks containing of the above medium and cultured at 27°C for 4 days on a rotary shaker. The broth filtrate (2.3 liters) was extracted with an equal volume of ethyl acetate. The active extract was concentrated to dryness under reduced pressure. The dried material (524.2 mg) was dissolved in a small volume of $CHCl_3$, and charged to a silica gel column. The column was first washed with $CHCl_3$ - MeOH (100 : 1) and then eluted with $CHCl_3$ - MeOH (100 : 2). The active material (114.5 mg) was further purified by reversed-phase HPLC using a Nucleosil C_{18} column with 20% MeOH to give a purified brownish powder (78.4 mg).

PTPase Assay

For preparation of the membrane fraction, Jurkat cells were homogenized in hypotonic lysis buffer (25 mM Tris-HCl, 25 mM sucrose, 0.1 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride and 10 μg/ml of leupeptin, pH 7.5), and centrifuged at 500 × g for 5 minutes. The supernatant was then centrifuged at 100,000 × g for 60 minutes, and the precipitate containing CD45 was dissolved in the assay buffer (100 mM sodium acetate and 1 mM EDTA; pH 6.0). Membrane preparation (2 μg protein) thus obtained and 1 mM *o*-phospho-L-tyrosine as a substrate in 45 μl of assay buffer with or without test sample were incubated at 37°C for 15 minutes. The reaction was terminated by addition of 5% $HClO_4$ (150 μl). For measurement of liberated inorganic phosphate, 50 μl of color reagent containing 6 N H_2SO_4, 1 mg/ml of malachite green, 2.5% of ammonium molybdate and 0.2% of Tween 20 was added to the mixture, and the absorbance at 620 nm was measured[9].

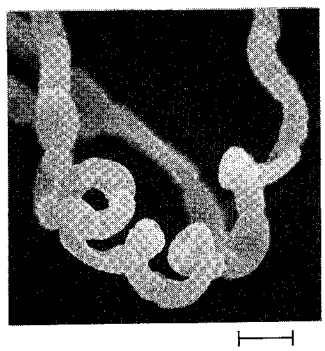
Results and Discussion

Taxonomic Characterization of the Producing Strain

The producing strain, MJ742-NF5, showed branched substrate mycelia, from which aerial hyphae developed in the form of open spirals. Sporangia and whirl-formation were not observed. Mature spore-chains usually bore more than 50 spores. Spores were 0.86~0.95 by 1.36~1.45 μm in size and had a smooth surface, as shown in Fig. 2.

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. MJ742-NF5.

Bar represents 1 μm.



The whole-cell hydrolysate of the strain contained LL-diaminopimelic acid. Based on these characteristics, strain MJ742-NF5 is considered to belong to the genus *Streptomyces*. Comparison of the strain with known series of *Streptomyces* showed that it is closely related to *S. roseosporus* and *S. roseolus*. Although the producing strain is spiral rather than rexiflexibile, other characteristics were the completely same in the 3 strains, as shown

Table 1. Comparison of taxonomic characterization of strain MJ742-NF5 with *Streptomyces roseosporus* and *Streptomyces roseolus*.

	MJ742-NF5	*Streptomyces roseosporus* IMC S-0143 (ISP 5122)		*Streptomyces roseolus* IMC S-0184 (ISP 5174)	
Spore chain	*Spirales*	*Rectiflexibiles*		*Rectiflexibiles*	
Spore surface	Smooth	Smooth		Smooth	
Spore size	0.86~0.95 × 1.36~1.45 μm	0.37~0.44 × 0.36~0.88 μm		0.56~0.63 × 1.06~1.25 μm	
Aerial mass color	Pale pink	Pale pink		Pale pink	
Color of growth	Pale yellowish-brown	Pale yellowish-brown		Pale yellowish-brown	
Soluble pigment	−	−		−	
Formation of melanoid pigment	−	−		−	
Liquefaction of gelatin	+	+		+	
Coagulation of milk	−	−		−	
Peptonization of milk	+	+		+	
Reduction of nitrate	+	+		+	
Hydrolysis of starch	+	+		+	
Utilization of[a]					
L-Arabinose	+	+	+[b]	+	+[b]
D-Xylose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	(−)	−	±	−	±
Rhamnose	+	+	+	+	+
Sucrose	−	−	−	−	−
Raffinose	−	−	−	−	−
Inositol	−	−	−	−	−
D-Mannitol	−	−	−	−	−

[a] +: Utilized, ±: doubtful, (−): probably not utilized, −: not utilized.
[b] Literature.

Fig. 3. Time course of dephostatin production.

○ Packed cell volume, □ inhibition of PTPase, ● pH.

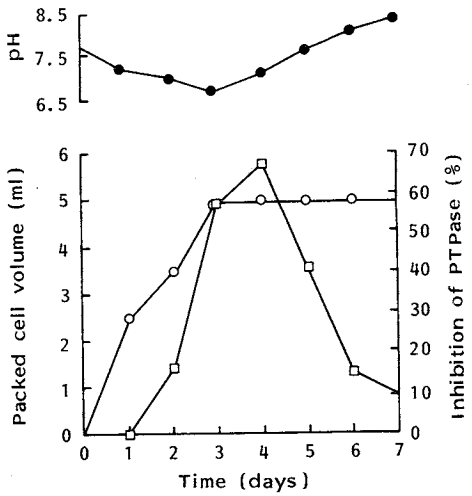


Fig. 4. Purification procedure for dephostatin.

Streptomyces sp. MJ742-NF5
↓ cultured for 4 days
Broth filtrate (2.3 liters)
↓ extracted with EtOAc
Organic phase
↓ silica gel chromatography, $CHCl_3$-MeOH
preparative HPLC, 20% MeOH
Dephostatin (78.4 mg)

in Table 1. Therefore, strain MJ742-NF5 was designated as *Streptomyces* sp. MJ742-NF5.

Production and Isolation of Dephostatin

The strain of *Streptomyces* sp. MJ742-NF5 was cultured in Erlenmeyer flasks at 27°C on a rotary shaker. The time course of the production is shown in Fig. 3. The maximum peak of dephostatin accumulation in the flasks was obtained at 4 days, thereafter the amount decreased. From the culture

filtrate (2.3 liters), 78.4 mg of dephostatin was isolated as shown in Fig. 4. The purity of each preparation was confirmed by TLC and HPLC.

Biological Activities of Dephostatin

As shown in Fig. 5, dephostatin inhibited PTPase activity with an IC_{50} of 7.7 μM. The IC_{50} value of sodium vanadate, a known PTPase and ATPase inhibitor[10], for this enzyme was 80 μM. Thus, inhibition of PTPase by dephostatin was 10-fold more potent than that by sodium vanadate in our assay system.

The Lineweaver-Burk plotting of PTPase with dephostatin showed that dephostatin was competitive with the substrate, and the *Ki* value of dephostatin obtained from the Dixon plot was 1.8×10^{-5} M, as shown in Fig. 6.

The growth inhibitory activities of dephostatin toward various cultured cells are shown in Table 2. Cytotoxicities of dephostatin on oncogene-expressing cells were similar to those on their parent normal cells. However, the Jurkat cells were more sensitive to dephostatin than the other cells tested. It is not

Fig. 5. Inhibitory activity of dephostatin toward protein tyrosine phosphatase.

○ Dephostatin, □ vanadate.

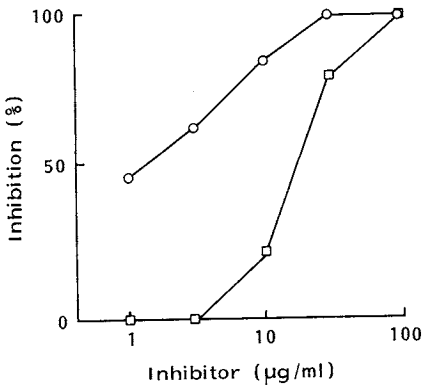
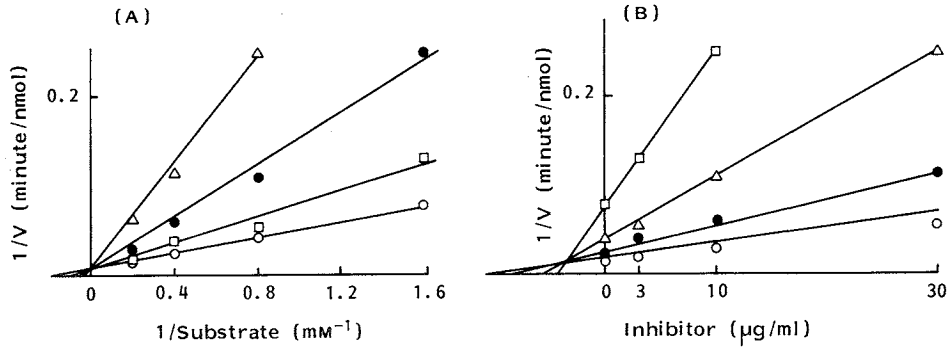


Table 2. Growth inhibition of cultured cells by dephostatin.

Cells		Cytotoxicity IC_{50} (μg/ml)
Jurkat		1.8
NRK		9.0
RSV^{ts}/NRK	(33°C)	8.1
	(39°C)	10.2
K-*ras*^{ts}/NRK	(33°C)	8.0
	(39°C)	8.9
NIH3T3		9.5
*erb*B2/NIH3T3		7.4
A431		7.3

Fig. 6. Inhibitory pattern of dephostatin.

(A) Lineweaver-Burk plot of PTPase reaction with dephostatin. Inhibitor concentrations are ○ 0, □ 3, ● 10, and △ 30 μg/ml. (B) Dixon plot of the PTPase reaction with dephostatin. Substrate concentrations are □ 0.63, △ 1.3, ● 2.5 and ○ 5 mM. The *Ki* value was obtained from the graph as 18 μM (3.0 μg/ml).



certain whether this effect is related to inhibition of PTPase.

Dephostatin also showed weak antibacterial activity with a MIC of 50~100 μg/ml. In an acute toxicity tested, the LD_{50} in ICR4W mice was greater than 100 mg/kg by intraperitoneal injection.

Acknowledgment

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