FORPHENICINE, AN INHIBITOR OF ALKALINE PHOSPHATASE PRODUCED BY ACTINOMYCETES

Sir:

Enzyme inhibitors can be powerful tools in analyzing the various aspects of homeostasis in living organisms and even in understanding disease processes. As we reported, small molecular inhibitors of endopeptidases, exopeptidases and glycosidases have been found in culture filtrates of actinomycetes[1~4].

Recently we demonstrated that activities of aminopeptidases, alkaline phosphatase and other enzymes are located on the surface of various types mammalian cells[5,6] and that these enzymes are indispensable in various functions of cells[7,8]. As reported previously, our experiments with bestatin[9,10], a specific inhibitor against aminopeptidase B and leucine aminopeptidase, demonstrated that it bound to FM3A and mouse spleen lymphocyte cells[8]. Bestatin enhanced delayed-type hypersensitivity and it produced immune resistance to experimental animal tumors[7,8,11]. We, therefore, searched for an inhibitor of alkaline phosphatase and found a new compound named forphenicine. As reported in another paper, forphenicine enhanced delayed-type hypersensitivity and increased the number of antibody-forming cells[7]. In this communication, we will report on the isolation and purification of forphenicine which inhibits alkaline phosphatase.

The method described by BESSEY[12] for determination of alkaline phosphatase (EC 3.1.3.1.) activity was modified for the quantitative assay of its inhibitors as follows[13]: to 0.02 ml of 0.1 M *p*-nitrophenyl phosphate (Daiichi Pure Chem. Co., Tokyo) in distilled water, 0.06 ml of 0.1 M carbonate buffer at pH 9.0, 0.01 ml of 0.2 M of magnesium chloride and 0.1 ml of distilled water with or without a test material were added; after 3 minutes at 37°C, 0.01 ml (0.25 μg of alkaline phosphatase of chicken intestine, Nutritional Biochemicals Co., U.S.A.) was added and the reaction mixture was incubated for 20 minutes at 37°C; after the incubation, the reaction was stopped by addition of 1.5 ml of 0.15 N NaOH and the extinction of the supernatant of the centrifuged solution was read at 400 nm. The reaction was also carried out without addition of enzyme solution and the result was taken as blank. The concentration of forphenicine required for 50% inhibition (IC_{50}) was calculated as described previously[14]. We observed that many strains of various species of actinomycetes produce an agent active in inhibiting alkaline phosphatase, and we isolated an active agent from the strain MC974–A5 which was isolated from the soil sample collected in Ito City, Shizuoka prefecture and was classified as *Actinomyces fulvoviridis* var. *acarbodicus*.

Forphenicine was produced by shaking culture or tank fermentations of the strain MC974–A5 in a medium containing soybean meal 1.5%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1%, K_2HPO_4 0.1%, $CuSO_4 \cdot 5H_2O$ 0.0007%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $MnCl_2 \cdot 4H_2O$ 0.0008%, $ZnSO_4 \cdot 7H_2O$ 0.0002% (pH was adjusted to 7.2 with 2 N NaOH before sterilization). The maximum production of forphenicine was attained on the 4~5th day of the shaking culture or on the 4th day of tank fermentation and maintained for 2~3 days thereafter.

Extraction and purification processes are shown in Chart 1. After 48-hour shaking culture at 27°C, the culture filtrate was adjusted to pH 3.0 with 6 N HCl and the precipitate was removed by centrifugation. The supernatant (24 liters) was adjusted to pH 5.0 with 6 N NaOH and passed through a column of carbon (6.5×20 cm). After the column was washed with 5 liters of distilled water, the adsorbed forphenicine was eluted with 50% ethanol. The active eluate (3.32 liters) was concentrated to 25 ml under reduced pressure and subjected to DEAE-Sephadex A–25 (formate

Chart 1. Isolation and purification of forphenicine

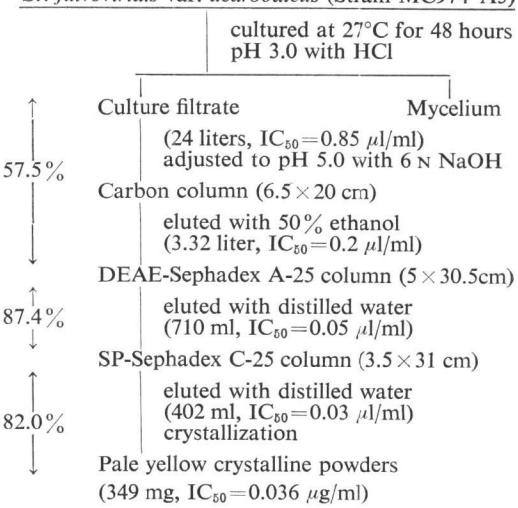

Fig. 1. Infrared absorption spectrum of forphenicine (KBr)

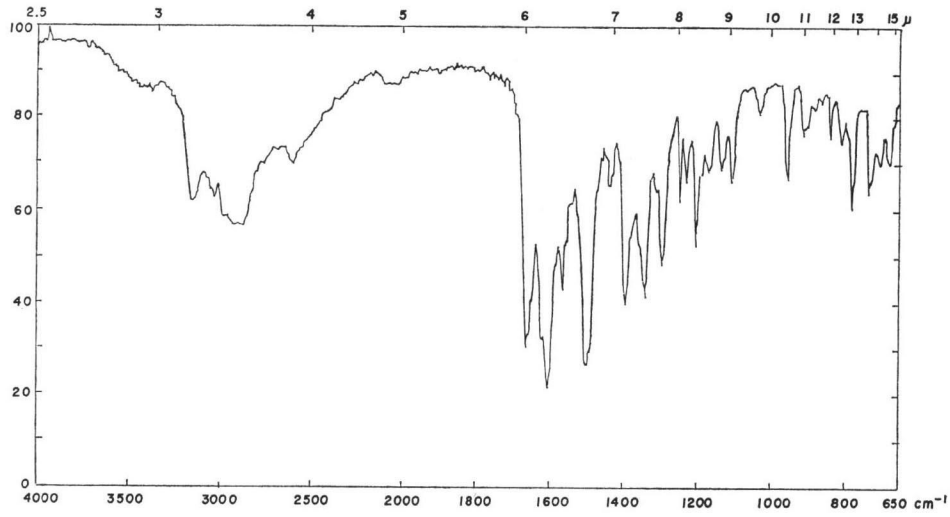

Table 1. Inhibitory activity of forphenicine on various alkaline phosphatase

	IC_{50} (μg/ml)						
	Chicken intestine	Hog intestine	Calf intestine	Calf liver	Calf kidney	Human placenta	*E. coli*
Forphenicine	0.036	>500	475	475	>500	>500	480
L-Phenylalanine	475	270	>500	>500	>500	220	>500

form, 5×30.5 cm) column chromatography using distilled water as the eluting solvent. The active eluate (710 ml) was concentrated to 10 ml under reduced pressure. Further purification of forphenicine was carried out on SP-Sephadex C-25 (H^+ form, 3.5×31 cm) column chromatography using distilled water as the eluant. Active fractions thus obtained were combined and dried. Forphenicine was recrystallized from water and it was obtained as pale yellow crystals.

Properties of forphenicine were as follows: m.p. >300°C; maxima at 258 nm ($E^{1\%}_{1cm}$ 780), 324 nm ($E^{1\%}_{1cm}$ 200) in 0.1 M phosphate buffer (pH 7.0). The IR spectrum is shown in Fig. 1. The elemental analysis was as follows: Calcd. for $C_9H_9NO_4$: C 55.38, H 4.65, N 7.18, O 32.79; found: C 55.81, H 4.79, N 7.05, O 32.22. The inhibitor is soluble in water and diluted acetic acid and insoluble in methanol, benzene, pyridine and dimethylsulfoxide. Forphenicine gave the following Rf value on silica gel thin-layer chromatography: 0.45 with *n*-butanol - acetic acid - water (3: 1: 1). The inhibitor moved to the cathode in formic acid - acetic acid - water (25: 75: 900, pH 1.9) on high voltage paper electrophoresis at 600 V for 30 minutes with an Rm value of 0.34, referring to L-alanine as 1.0. The structure of forphenicine was determined as 4-formyl-3-hydroxy-phenylglycine. The structure study were reported in the accompanying paper[15].

Table 1 shows the inhibitory activities of forphenicine against alkaline phosphatases of various origins. The inhibitory effect of L-phenylalanine[16,17], which has been reported to inhibit alkaline phosphatase from certain organs, was also compared. Forphenicine showed potent inhibition against the enzyme prepared from chicken intestine and slightly inhibited the enzymes prepared from calf intestine, liver and *Escherichia coli*. Other phosphatases were not affected. The type of inhibition of chicken intestine enzyme by forphenicine was uncompetitive with *p*-nitrophenyl phosphate and its Ki value was 1.64×10^{-7} M. Forphenicine at 100 μg/ml demonstrated no inhibition of the growth of Gram-positive and Gram-negative

bacteria in a nutrient medium. Forphenicine has low toxicity. Forphenicine was not so soluble in water and intravenous injection of 125 mg/kg of forphenicine did not cause death of mice.

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TAKAAKI AOYAGI
TAKUZO YAMAMOTO
KATSUHISA KOJIRI
FUKIKO KOJIMA
MASA HAMADA
TOMIO TAKEUCHI
HAMAO UMEZAWA

Institute of Microbial Chemistry
Kamiosaki, Shinagawa-ku, Tokyo
Japan

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