Communications to the Editor

ALPHOSTATIN, AN INHIBITOR OF ALKALINE PHOSPHATASE OF BOVINE LIVER PRODUCED BY *BACILLUS MEGATERIUM*

Sir:

Previously, we have shown that activities of aminopeptidases, carboxypeptidases, alkaline phosphatase and esterase were located not only in cells but also on the cellular membrane of various kinds of mammalian cells[1~4]. We have succeeded in obtaining inhibitors against these enzymes from culture filtrates of the actinomycetes, bestatin, amastatin, forphenicine, esterastin, ebelactones A and B and so forth[5~9]. Among these inhibitors, bestatin, amastatin, forphenicine, ebelactones A and B enhanced immune responses, but esterastin suppressed immune responses[10,11]. These inhibitors were chosen as objects of study by many because of their interesting biological activities.

In screening of inhibitors of alkaline phosphatase in bovine liver (E.C. 3.1.3.1), we have recently isolated a new one from the culture filtrate of *Bacillus megaterium* BMG59-R2 which was isolated from the soil sample collected in Institute of Microbial Chemistry. In this communication, we report the isolation and characterization of alphostatin and its activity.

In determining the inhibitory activity against alkaline phosphatase, the method used previously[7] was modified as follows: 0.04 ml of 0.1 M sodium *p*-nitrophenyl phosphate (Daiichi Pure Chem. Co., Tokyo), 0.02 ml of 0.2 M magnesium chloride, 0.32 ml of buffer (0.3 M 2-amino-2-methyl-1,3-propandiol HCl) at pH 9.0 and 0.1 ml of distilled water, with or without a test material, was added; after the incubation for 3 minutes at 37°C, 0.02 ml of alkaline phosphatase solution (bovine liver, Sigma Chemical Company, Saint Louis) was added and the reaction mixture was incubated for 20 minutes at 37°C. 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 420 nm. The concentration of enzyme in the reaction mixture was adjusted to yield 50 nmol of *p*-nitrophenol. The reaction was also carried out without the enzyme solution to obtain the blank value and the concentration of the inhibitor required for 50% inhibition (IC_{50}) was determined.

Alphostatin was produced by shaking culture of the strain BMG59-R2 in media containing various carbon and nitrogen sources. Carbon sources included glycerol, glucose, lactose, maltose and starch, while nitrogen sources included fish meal, NZ-Amine, yeast extract and soybean meal for the production of alphostatin. A typical medium used for production contained glycerol 3.0%, casein 3.0%, NaCl 0.1%, K_2HPO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.3% (pH 7.6). Maximum production was attained in 25~30 hours in the shaking culture and maintained for 48 hours thereafter.

Alphostatin in 30 liters of a culture filtrate was applied on Diaion PK-216 (H^+, 2×46 cm), and the effluent was adsorbed on activated carbon. The column was washed with distilled water and was eluted with 6 liters of 80% methanol. The eluate was concentrated to a syrup under a reduced pressure and was dissolved in 500 ml of water. The solution was applied to a DEAE-Sephadex A-25 column (formate, 5× 40 cm). The active fraction obtained by elution with a linear gradient to 0.5 M sodium formate solution and applied to Diaion PK-216 (H^+, 3×15 cm) column. The effluent was concentrated to dryness. The residue was dissolved in water and applied to a DEAE-Sephadex A-25 (3×90 cm) and the active fraction obtained by elution with a linear gradient to 0.5 M sodium formate solution. The crude powder (500 mg) thus obtained had a potency of 4 μg/ml (IC_{50}). For further purification, the above-mentioned DEAE-Sephadex A-25 and Diaion PK-216 column chromatographies were repeated.

Alphostatin was obtained as a colorless crystalline powder (100 mg, IC_{50} 2 μg/ml) and it had the following properties: MP 203~205°C (dec); no characteristic UV adsorption. The IR spectrum is shown in Fig. 1. The elemental analysis was as follows: Calcd for $C_{25}H_{45}N_6O_{13}P \cdot H_2O$: C 43.73, H 6.90, N 12.24; found: C 43.46, H 6.91, N 12.17. Alphostatin is soluble in water and diluted hydrochloric acid, but insolu-

Fig. 1. IR spectrum of alphostatin.

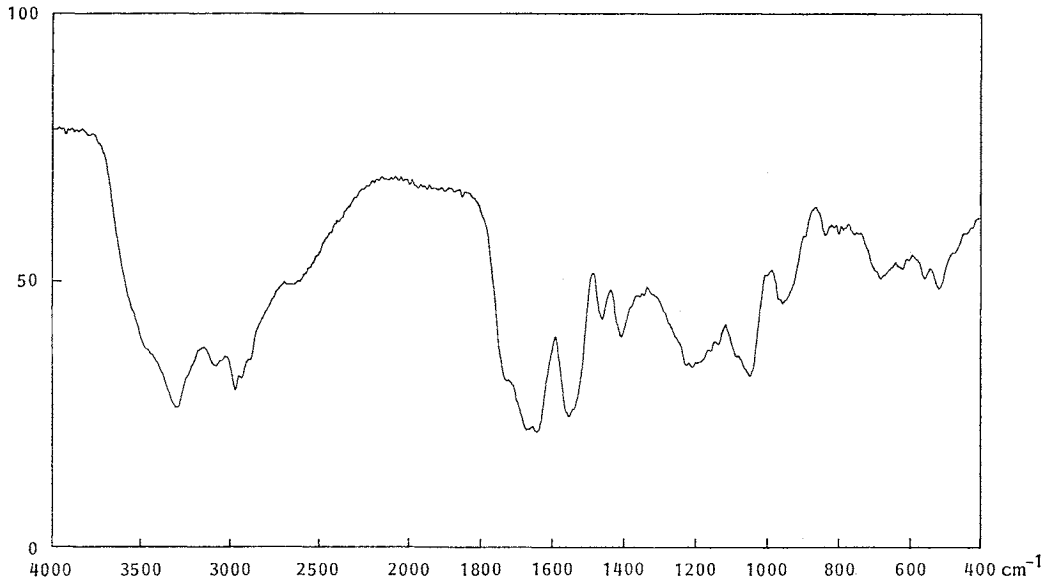

Table 1. Inhibitory activity of alphostatin and forphenicine on various alkaline phosphatase.

Inhibitor	IC_{50} (M)				
	Bovine liver	Bovine intestine	Human placenta	Chicken intestine	*Escherichia coli*
Alphostatin	3.0×10^{-6}	$>1.5\times10^{-4}$	$>1.5\times10^{-4}$	$>1.5\times10^{-4}$	$>1.4\times10^{-4}$
Forphenicine	2.4×10^{-3}	$>2.6\times10^{-3}$	$>2.6\times10^{-3}$	1.8×10^{-7}	$>2.6\times10^{-3}$

ble in acetone, benzene and ethyl acetate. It gave positive Rydon-Smith, ninhydrin and acid molybdate reactions and negative Pauly, Ehrlich, resorcinol-HCl and 2,4-dinitrophenylhydrazine reactions. On silica gel (E. Merck) TLC, it gave a single spot at Rf 0.21 with butanol - acetic acid - water (3 : 1 : 1). It moves toward the cathode in formic acid - acetic acid - water (25 : 75 : 900) under 600V paper electrophoresis for 60 minutes showing Rm value 0.058 (alanine=1). As will be reported in the next paper, the structure of inhibitor was determined to be L-isoleucyl-L-isoleucyl-L-phosphoseryl-L-glutaminyl-L-glutamic acid[12], which showed IC_{50} value of 3.0×10^{-6} M.

In the course of the structure determination study, we synthesized a partial peptide, L-isoleucyl-L-isoleucyl-L-phosphorylserine, which showed IC_{50} value of 6.3×10^{-6} M. This result suggested that L-glutamyl-L-glutamic acid moeity of the inhibitor is not essential for its inhibitory activity.

Activities of alphostatin in inhibiting various kind of alkaline phosphatases are shown in Table 1 in comparison with forphenicine. Alphostatin showed potent inhibition against the enzyme prepared from bovine liver but did not affect other phosphatases.

At the concentration of 100 μg/ml alphostatin had no antimicrobial activity. It has low acute toxicity. No deaths occurred after iv injection of 250 mg/kg of this agent to mice.

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