A THERMOLYSIN INHIBITOR PRODUCED BY ACTINOMYCETES: PHOSPHORAMIDON

Sir:

As reported in previous papers and as described in a monograph[1], microorganisms produce inhibitors of proteases. Leupeptin[2,3] and antipain[4,5] inhibit trypsin, plasmin and papain, chymostatin[6] inhibits chymotrypsin and weakly papain, and pepstatin[7~10] inhibits pepsin, gastricsin, cathepsin D and renin. Cathepsin B is inhibited by leupeptin[11], and cathepsins A and B are inhibited by antipain[12]. Our search for an inhibitor of thermolysin yielded phosphoramidon (I) which had been previously discovered as an EHRLICH reaction-positive compound in a culture filtrate[13].

Thermolysin (EC 3.4.4 group) is a metalloendopeptidase isolated from the culture filtrate of a thermostable organism, *Bacillus thermoproteolyticus*. The specificity of this enzyme is directed to a peptide bond in which the amino group of hydrophobic amino acids such as leucine, isoleucine and phenylalanine[14] are involved, and X-ray study has shown the three dimensional structure of this enzyme containing a zinc ion at the center of the cleft[15].

An inhibitor of thermolysin was isolated from culture filtrates of various species of actinomycetes. With strain MD706-Y4, *Streptomyces tanashiensis* its production and isolation were studied in detail. The concentration of the inhibitor was determined by its thermolysin-inhibiting activity. Thermolysin was measured by the following method. The reaction mixture contained 0.9 ml of 0.1 M Tris-HCl buffer at pH 7.5 containing 0.01 M $CaCl_2$ and 0.06 M NaCl with or without the test material, 1.0 ml of 2% casein solution adjusted to pH 7.5 with 1 N NaOH, and 0.1 ml of a 7.5 μg/ml enzyme solution. The reaction mixture without the enzyme solution was first incubated for 3 minutes at 37°C and the reaction was started by addition of the enzyme solution. After incubation at 37°C for 30 minutes, 2.0 ml of 1.7 M perchloric acid was added and the mixture kept for 1 hour at room temperature. After centrifugation, the extinction of the acid soluble fraction was read at

Fig. 1. Chromatography on DEAE-Sephadex A-25
Bed volume: 100 ml
Eluent: 1 M CH_3COOH+NaCl 0~1.0 M (linear); 1,000 ml
Each fraction: 7 g

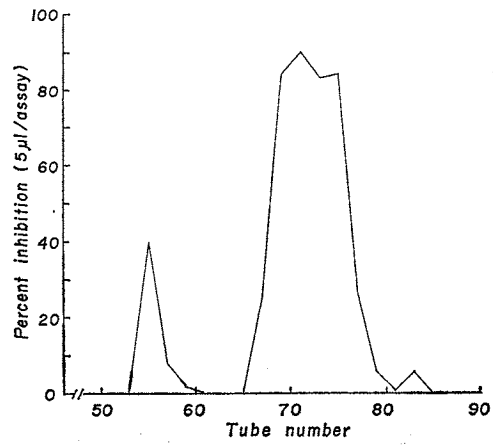


Fig. 2. Infrared absorption spectrum of phosphoramidon sodium salt (KBr)

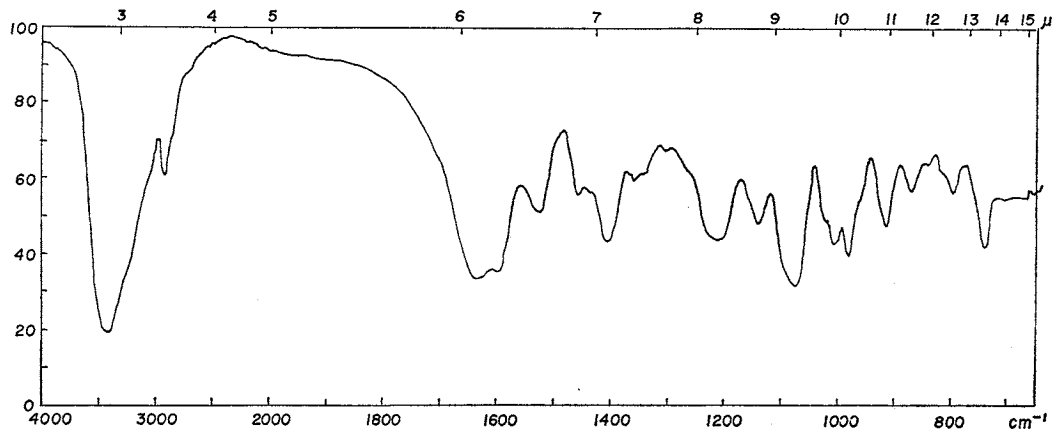
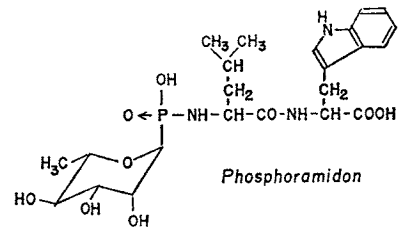


Table 1. Inhibitory activity of phosphoramidon on various proteases

	ID_{50} (μg/ml)				
	Trypsin	Papain	α-Chymotrypsin	Pepsin	Thermolysin
Phosphoramidon	>250	>250	>250	>250	0.4



280 nm. The reaction was also carried out in the reaction mixture without the enzyme solution and the result was taken as the blank value. The concentration of the inhibitor required for 50% inhibition (ID_{50}) was calculated as described in a previous paper[3].

The inhibitor was produced by the rotary culture or by tank fermentation of the strain MD706-Y4 in media containing various kinds of carbon sources and nitrogen sources. For example it was produced in a medium containing 2.5% glycerol, 0.5% meat extract, 0.5% polypeptone, 1.0% yeast extract, 0.2% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% K_2HPO_4 and 0.32% $CaCO_3$ adjusted to pH 7.4 with 2 N NaOH. Maximum production was attained in 2~3 days by rotary culture and maintained for 2~4 days thereafter.

The inhibitor in the culture filtrate was adsorbed on activated carbon and eluted with methanol at pH 8.0. The active elute was evaporated under reduced pressure and the powder thus obtained was dissolved in 1 M acetic acid and purified by DEAE-Sephadex A-25 column chromatography with a linear gradient of NaCl from 0~1.0 M in 1 M acetic acid. The active fractions were combined and evaporated under reduced pressure and the residue was extracted with ethanol. The powder thus obtained was dissolved in distilled water and neutralized with 0.1 N NaOH. Evaporation of the solution under reduced pressure gave the sodium salt of the inhibitor. It showed 50% inhibition of thermolysin at 0.5 μg/ml. Further purification, using the methanol solution of the sodium salt for Sephadex LH-20 chromatography, gave the completely purified compound.

Properties of the sodium salt of the inhibitor was as follows: m.p. 173~178°C (dec.); $[\alpha]_D^{20}$ −33.6° (*c*. 1.0, H_2O); maxima at 221 nm ($E_{1\,cm}^{1\%}$ 480), 275 nm (sh.) ($E_{1\,cm}^{1\%}$ 76), 282 nm ($E_{1\,cm}^{1\%}$ 81), 289.5 nm ($E_{1\,cm}^{1\%}$ 69.5) in H_2O. The IR spectrum is shown in Fig. 2. The elemental analysis was as follows: calcd. for $C_{23}H_{32}N_3O_{10}P_1Na_2$: C 47.02, H 5.49, N 7.15, O 27.23, P 5.27, Na 7.82. Found: C 46.98, H 5.46, N 7.11, O 27.30, P 5.44. It is soluble in water, methanol and dimethylsulfoxide, less soluble in ethanol, ethylacetate, insoluble in benzene, hexane, petroleum ether, ethyl ether and chloroform. It gave positive EHRLICH, ammonium molybdate-perchloric acid and RYDON-SMITH reactions and negative ninhydrin reaction. It gave the following Rf values in thin-layer chromatography: 0.32 with *n*-butanol-acetic acid-water (4:1:1) on silica-gel, 0.67 with *n*-butanol-acetone-acetic acid-5% ammonia-water (35:25:15:15:10) on cellulose (Avicel). It moved to the anode in formic acid-acetic acid-water (25:75:900) electrophoresis at 3,500 V for 15 minutes with an Rm value of −0.32 taking L-alanine as 1.0. The identity of the compound here obtained with phosphoramidon was confirmed by comparison with an authentic sample obtained by S. UMEZAWA *et al.*[13]

The inhibition of proteases by phosphoramidon was examined with the results shown in Table 1. The testing methods have been reported in papers[3] on leupeptin against trypsin, papain, chymotrypsin and in another[8] on pepstatin against pepsin. The results shown in Table 1 indicate that phosphoramidon is a specific inhibitor of metallo-endopeptidase. This specific action implies an interesting structural relation of phosphoramidon (*N*-(α-L-rhamnopyranosyloxyhydroxyphosphinyl)-L-leucyl-L-tryptophan) with thermolysin. Zn^{++} is located in the active site of this enzyme and phosphoric acid moiety which links to leucine moiety in phosphoramidon may interact Zn^{++} in the enzyme. It suggests a role for the phosphoryl amino acid group in the inhibition of a metallo-enzyme which is specific in cleavage of the amino group side of an amino acid residue in a peptide. Phosphoramidon at 100 μg/ml showed no antibacterial and no antifungal activity. It has low toxicity and the intravenous injection of 1.0 g/kg to mice caused no death.

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