Short Communication

New Metallo Proteinase Inhibitor
(MK-I) Produced by *Streptomyces*
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In the course of investigations of proteinase inhibitors in microbes,^{1~4)} a new strain was isolated from soil which produces a potent metallo proteinase inhibitor in culture filtrate. The strain was identified as a new species of genus *Streptomyces* by its morphological properties and named *Streptomyces mozunensis* MK-23. The inhibitor was designated as MK-I.

For the production of MK-I, *Streptomyces mozunensis* MK-23 was cultured in a medium containing 1% glycerol, 4% polypepton, 0.1% K_2HPO_4 , 0.1% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0001% $CuSO_4 \cdot 5H_2O$, 0.0001% $ZnSO_4 \cdot 7H_2O$ and 0.0001% $MnSO_4 \cdot 6H_2O$ (pH 7.0). After cultivation for 22 to 25 hr at 30°C, the culture filtrate (containing about 200 mg/liter of MK-I) was mixed with carbon. The carbon was washed with water and then with 70% methanol containing 0.1 M ammonia. The methanolic washings were evaporated to dryness *in vacuo*, and the residue was dissolved in water. The solution was adjusted to pH 2 at 4°C and extracted with an equal volume of *n*-butanol. The extract was adjusted to pH 7 and re-extracted with water. The aqueous extract was evaporated *in vacuo*, and the residue was dissolved in 1 M acetic acid at 4°C. The solution was applied to a column of DEAE-Sephadex A-25, previously equilibrated with 1 M acetic acid. The elution was carried out with a linear gradient of NaCl from 0 to 0.5 M in 1 M acetic acid. The active fractions were collected and applied

to a carbon column. The column was washed with water and eluted with 90% methanol containing ammonia (pH 10.5). The active fractions were collected and evaporated to dryness *in vacuo*. The residue was dissolved in a minimal volume of water. The solution was applied to a column of Sephadex G-10, and the column was eluted with water. The active fractions were collected and lyophilized to give pure MK-I in a white powder form. The overall yield of MK-I was about 20%.

MK-I so obtained gave a single spot on thin layer chromatography with Kieselgel 60 (E. Merck). *R_f* values were 0.41 and 0.49 in *n*-butanol-acetic acid-water (4:1:1) and *n*-butanol-pyridine-acetic acid-water (15:10:3:12), respectively. High performance liquid chromatography of MK-I gave a single peak on LiChrosorb RP-18 (E. Merck) with 40% methanol (pH 7.0). Thus MK-I was proved to be homogeneous. Properties of MK-I were as follows: mp 146~147°C (dec.), dirubidium salt 193~195°C (dec.); $[\alpha]_D^{25} -26.9^\circ$ (dirubidium salt, $c=0.57$, H_2O); Elemental Analysis (dirubidium salt); Found: C, 34.64; H, 5.20; N, 5.22. Calcd. for $C_{22}H_{32}N_3O_{10}PRb_2 \cdot 5H_2O$: C, 34.42; H, 5.27; N, 5.24%; UV: $\lambda_{max}^{H_2O} (\epsilon)$ 278 (5700); IR: $\nu_{max}^{KBr} cm^{-1}$; 3400~3100 (OH, NH), 2950 (CH), ~1640 (amide I), 1590 (phenyl), 1520 (amide II), 1400 (CH), 1200 (P=O), ~1070 (C-O), 740 (phenyl). 1H -NMR (100 MHz, in D_2O): shown in Fig. 1. MK-I was soluble in water and alcohol, insoluble in benzene, ether and chloroform. Its color reaction was positive to Rydon-Smith, Ehrlich, molybdate-perchloric acid and diphenylamine-aniline reagents, and negative to ninhydrin. MK-I was stable in neutral or alkaline solution, but less stable in acidic solution.

On hydrolysis of MK-I, leucine and tryptophan were liberated with 4 N NaOH (110°C, 48 hr), and leucine and a trace of tryptophan with 6 N HCl (110°C, 48 hr). NMR data also showed that MK-I had leucine and tryptophan. Mild acid treatment (pH 1, r.t. 24 hr) of MK-I gave sugar, phosphoric acid and peptide. NMR data suggested that the sugar was 6-deoxyhexose. High performance liquid and gas

FIG. 1. Proton WEFT-NMR Spectrum of MK-I (100 MHz, in D₂O).

chromatographies suggested that the sugar was 6-deoxytalose. The peptide moiety of MK-I was identified as L-leucyl-L-tryptophan by comparison with the authentic sample. Thus the structure of MK-I is *N*-(6-Deoxytalosyloxyphosphonyl)-L-leucyl-L-tryptophan.

The inhibitory activity of MK-I was tested against various proteases. Thermolysin inhibiting activity of MK-I was measured by the following method. A mixture of 0.25 ml of thermolysin solution (20 μ g/ml in 0.05 M phosphate buffer at pH 7.5) and 0.25 ml of MK-I solution (in the same buffer) was pre-incubated for 10 min at 37°C. The mixture was supplemented with 1.5 ml of 1.33% casein solution in 0.1 M phosphate buffer (pH 7.0) and incubated for 10 min at 37°C. After the addition of 2.0 ml of 0.44 M trichloroacetic acid and standing for 20 min, the reaction mixture was filtered. To 10 ml of the filtrate 5.0 ml of 0.44 M Na₂CO₃ and 1.0 ml of 2-fold diluted Folin-Ciocalteu reagent were added. After the mixture was allowed to stand for 20 min at 37°C, the absorbance was measured at 660 nm. The blank or control values were taken by carrying out the reaction without enzyme or inhibitor. Inhibitory activity against other proteases was measured under the optimum conditions for each enzyme, and the results are summarized in Table I. Thermolysin and

the metallo proteinase of *Pseudomonas aeruginosa* IFO-3455 (named as elastase)^{b)} were strongly and carboxypeptidase A was very weakly inhibited by MK-I. On the other hand, trypsin, chymotrypsin, papain and pepsin were not inhibited. This result shows that MK-I is a specific inhibitor of metallo proteases.

TABLE I. INHIBITORY SPECTRUM OF MK-I AGAINST VARIOUS PROTEASES

Inhibitory activity was estimated by measuring residual enzyme activity after incubation with MK-I (5 μ g) at 37°C for 10 min. +, More than 70% inhibition; $\pm$, less than 10% inhibition; -, no inhibition of enzyme activity.

Protease		Inhibition
Thermolysin	(5 μ g)	+
Metallo proteinase of <i>Ps. aeruginosa</i> IFO-3455	(3.5 μ g)	+
Carboxypeptidase A	(10 μ g)	$\pm$
Trypsin	(25 μ g)	-
Chymotrypsin	(25 μ g)	-
Papain	(25 μ g)	-
Pepsin	(25 μ g)	-

Phosphoramidon^{6,7)} produced by *Streptomyces tanashiensis* is known as an inhibitor of thermolysin and the elastase of *Pseudomonas aeruginosa*. The sugar moiety of MK-I is different from that of phosphoramidon, which

is composed of rhamnose, phosphoric acid and leucyltryptophan. Another known metallo protease inhibitor S-MPI⁴⁾ has a molecular weight of 11,000~12,000. Thus MK-I is different from these inhibitors reported so far.

It is concluded that MK-I is a novel inhibitor of metallo protease. Details of this work will be reported later.

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