ELASTATINAL, A NEW ELASTASE INHIBITOR PRODUCED BY ACTINOMYCETES

Sir:

As reported in previous papers, protease inhibitors such as leupeptin, antipain, chymostatin, pepstatin and phosphoramidon have been obtained from cultured broth of actinomycetes.[1] Leupeptin[2,3] and antipain[4] inhibit trypsin, plasmin and papain; chymostatin[5] inhibits chymotrypsin; pepstatin[6~8] inhibits pepsin, gastricsin, cathepsin D and renin; and phosphoramidon[9] inhibits metallo-endopeptidase, such as thermolysin.

Elastase (EC 3.4.4.7) reacts with elastin in the elastic tissue and hydrolyzes the peptide bonds involving the carbonyl groups of amino acids bearing uncharged nonaromatic side chains, such as alanine[10,11]. In this communication, isolation of elastatinal which inhibits elastase is reported.

Elastatinal was found in culture filtrates of various species of actinomycetes, one of which (strain MD 469-CG8) was used for detailed production and isolation studies. Strain MD469-CG8 was isolated from a soil sample collected in Kumamoto Prefecture and found to be closely related to *Streptomyces griseoruber*.

In order to obtain quantitative values of anti-elastase activity, the method described by NAUGHTON *et al.*[11] was modified as follows: to 1.0 ml of 0.2 % elastin-Kongored (Boehringer Ltd., Mannheim, Germany) in 0.2 M Tris-HCl buffer (pH 8.8) was added 0.98 ml of the same buffer with or without a test material; after 3 minutes at 37°C, 0.02 ml (2 μg of elastase in the same buffer) was added and the reaction mixture incubated for 30 minutes at 37°C; after the incubation, 2.0 ml of 0.5 M potassium phosphate buffer (pH 6.0) was added, and the extinction of the supernatant of the centrifuged reaction mixture was read at 492 nm. The reaction was also carried out in the reaction mixture without the enzyme solution to obtain the blank value. The concentration of the inhibitor for 50 % inhibition (ID_{50}) was calculated as described in a previous paper.[3]

Elastatinal was produced by shaking culture or tank fermentation of the strain MD469-CG8 in media containing various kinds of carbon sources and nitrogen sources. For example it was produced in a medium containing 3.0 % glucose, 2.0 % soybean meal, 0.3 % NaCl, 0.25 % NH_4Cl, 0.6 % $CaCO_3$, adjusted to pH 7.2 with 2 N NaOH, with maximum production attained after 48~66 hours on a rotary shaking machine or 38~48 hours by the tank fermentation.

Elastatinal in a culture filtrate was adsorbed on active carbon and eluted with 50 % acetone. The active eluate was evaporated under reduced pressure and passed through a column of Dowex 1×2 (acetate form). The effluent and water used for washing were mixed and adjusted to pH 3.1 with formic acid. This solution was chromatographed on SP-Sephadex C-25 which was equilibrated with 50 mM pyridine-formic acid buffer at pH 3.1, and eluted with 75 mM pyridine-formic acid buffer at pH 4.1. The active fractions were combined and evaporated under reduced pressure. Repetition of the SP-Sephadex chromatography gave purified elastatinal, which showed 50 % inhibition of elastase at 1.8 μg/ml.

Properties of elastatinal was as follows: m.p. 196~204°C (dec.); $[\alpha]_D^{25}$ +2° (*c* 1.0, H_2O); a very weak maximum at 275 nm ($E_{1cm}^{1\%}$ 1.5) in 0.1 N HCl, 298 nm ($E_{1cm}^{1\%}$ 2.5) in 0.1 M phosphate buffer at pH 7.0. It was unstable in alkaline solution, and it showed a maximum at 304 nm ($E_{1cm}^{1\%}$ 78.8) in 0.1 N NaOH. The IR spectrum is shown in Fig. 1.

Found: C45.94, H 7.13, N 19.92.

Calcd. for $C_{21}H_{36}N_8O_7 \cdot 2H_2O$: C 45.97, H 7.34, N 20.42.

It was soluble in water, methanol, pyridine, dimethylsulfoxide, slightly soluble in ethanol, *n*-propanol, acetone, chloroform and insoluble in *n*-butanol, ethyl acetate, butyl acetate, hexane, toluene, benzene and ethyl ether. It gave positive RYDON-SMITH, FOLIN, triphenyltetrazolium chloride, 2, 4-dinitrophenyl hydrazine, nitroprusside-ferricyanide and silver nitrate-sodium hydroxide, and negative ninhydrin, SAKAGUCHI, EHRLICH, and anthrone reactions. It gave the following Rf values in silica gel thin-layer chromatography: 0.31 with *n*-butanol - acetic acid - water (4:1:1), 0.60 with methanol - pyridine-water (20:1:5). It moved toward the cathode in formic acid - acetic acid - water (25:75:900) under 3,500 V electrophoresis for 15 minutes with an Rm value of 0.58 taking alanine as 1.0. It showed two pKá values:

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

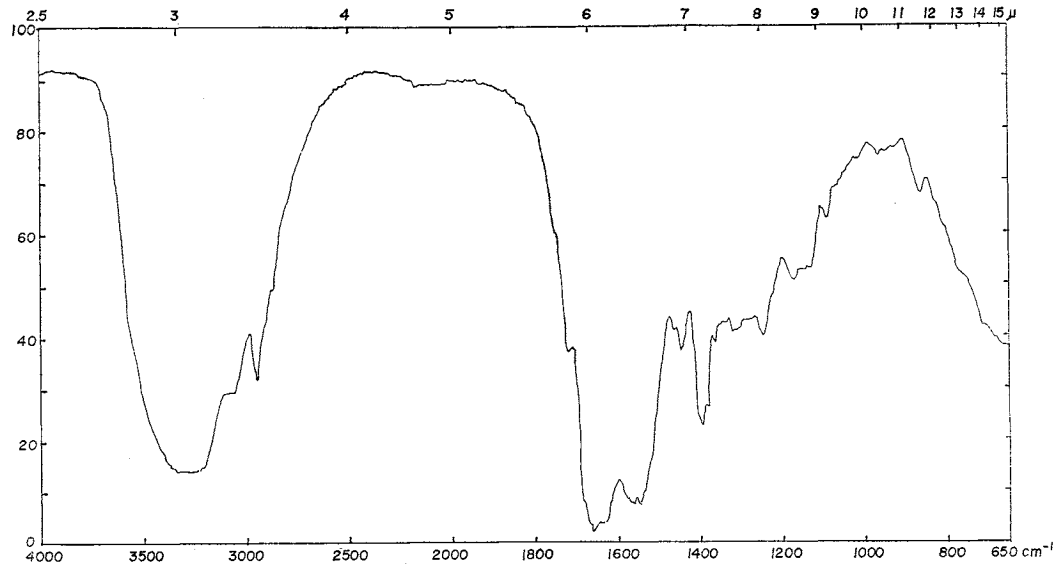

Table 1. Inhibitory activity of elastatinal on various proteases.

	ID_{50} (μg/ml)						
	Elastase	Trypsin	Plasmin	Papain	Chymotrypsin	Thermolysin	Pepsin
Elastatinal	1.8	>250	>250	>250	>250	>250	>250

3.7, >10.5 (titration equivalent 508). Elastatinal was hydrolyzed with 6 N HCl at 110°C for 24 hours. Amino acid analysis indicated the presence of one mole of glutamic acid, 0.2 mole of leucine and an unusual basic amino acid, the structure of which will be reported shortly. Elastatinal at 100 μg/ml showed no antibacterial and no antifungal activity. It has low toxicity and the intravenous injection of 250 mg/kg to mice caused no death. The activities of elastatinal against elastase and the other proteases are shown in Table 1. The methods employed for testing these activities have been described in previous papers.[7] The results shown in Table 1 indicate that elastatinal is a specific inhibitor of elastase. This specific action is interesting with regard to the structural relationship of elastatinal to elastase. As will be reported, the C-terminal residue of elastatinal is alaninal. It has been shown that the C-terminal residue of leupeptin which inhibits trypsin is argininal and the C-terminal residue of chymostatin[12] which inhibits chymotrypsin is phenylalaninal. The structures of these inhibitors obtained from microbial culture filtrates thus show a consistent structural feature involved in protease inhibition. In this connection, one should note THOMPSON's[13] paper which appeared after the report on the leupeptin structure and synthesis of chymotrypsin inhibiting peptides[14] and which reports the synthesis of Ac-Pro-Ala-Pro-alaninal as an elastase inhibitor.

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References

1) UMEZAWA, H.: Enzyme inhibitors of microbial origin. University of Tokyo Press, 1972
2) AOYAGI, T.; T. TAKEUCHI, M. MATSUZAKI, K.

KAWAMURA, S. KONDO, M. HAMADA, K. MAEDA & H. UMEZAWA: Leupetins, new protease inhibitors from actinomycetes. J. Antibiotics 22:283~286, 1969

3) AOYAGI, T.; S. MIYATA, M. NANBO, F. KOJIMA, M. MATSUZAKI, M. ISHIZUKA, T. TAKEUCHI & H. UMEZAWA: Biological activities of leupeptins. J. Antibiotics 22:558~568, 1969
4) SUDA, H.; T. AOYAGI, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Antipain, a new protease inhibitor isolated from actinomycetes. J. Antibiotics 25:236~266, 1972
5) UMEZAWA, H.; T. AOYAGI, H. MORISHIMA, S. KUNIMOTO, M. MATSUZAKI, M. HAMADA & T. TAKEUCHI: Chymostatin, a new chymotrypsin inhibitor produced by actinomycetes. J. Antibiotics 23:425~427, 1970
6) UMEZAWA, H.; T. AOYAGI, H. MORISHIMA, M. MATSUZAKI, M. HAMADA & T. TAKEUCHI: Pepstatin, a new pepsin inhibitor produced by actinomycetes. J. Antibiotics 23:259 ~ 262, 1970
7) AOYAGI, T.; S. KUNIMOTO, H. MORISHIMA, T. TAKEUCHI & H. UMEZAWA: Effect of pepstatin on acid proteases. J. Antibiotics 24:687~694, 1971
8) AOYAGI, T.; H. MORISHIMA, R. NISHIZAWA, S. KUNIMOTO, T. TAKEUCHI & H. UMEZAWA: Biological activity of pepstatins, pepstanone A and partial peptides on pepsin, cathepsin D and renin. J. Antibiotics 25:689~694, 1972
9) SUDA, H.; T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: A thermolysin inhibitor produced by actinomycetes: Phosphoramidon. J. Antibiotics 26: 621~623, 1973
10) SHOTTON, D.M.: Proteolytic enzymes. Methods in Enzymology 19:113~140, 1970
11) NAUGHTON, M.A. & F. SANGER: Purification and specificity of pancreatic elastase. Biochem. J. 78:156~163, 1961
12) TATSUTA, K.; N. MIKAMI, K. FUJIMOTO, S. UMEZAWA, H. UMEZAWA & T. AOYAGI: The structure of chymostatin, a chymotrypsin inhibitor. J. Antibiotics 26:625~646, 1973
13) THOMPSON, R.C.: Use of peptide aldehydes to generate transitionstate analogs of elastase. Biochemistry 12:47~51, 1973
14) ITO, A.; K. TOKAWA & B. SHIMIZU: Peptide aldehydes inhibiting chymotrypsin. Biochem. Bopihys. Res. Comm. 49:343~349, 1972