Communications to the editors

LEUPEPTINS, NEW PROTEASE INHIBITORS FROM ACTINOMYCETES

Sir:

Antiplasmin activity of culture filtrates of actinomycetes were examined and the active compounds were isolated. The compounds were acetyl- or propionyl-L-leucyl-L-leucyl-DL-argininal, and their analogues which contained isoleucine or valine instead of one or two leucines and they were named leupeptins.

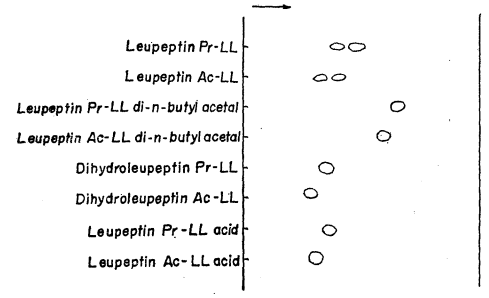
Ten strains which produced leupeptins were examined in detail and classified as follows: the strains MA 839-A1, MB 262-M1, *Streptomyces roseus*; MA 943-M1, MB 260-A2, MB 456-AE1, *S. roseochromogenes*; MB 58-MG1, *S. chartreusis*; MB 26-A1, *S. albireticuli*, MB 321-A1, *S. thioluteus*; MB 172-A2, *S. lavendulae*; MB 46-AG1, *S. noboritoensis*. Leupeptins were found also in culture filtrates of other strains which could be classified as more than 11 other species. Thus, leupeptins are widely produced by actinomycetes.

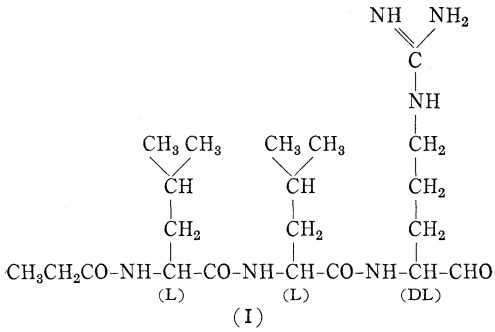
The method of testing the antiplasmin activity was as follows: a mixture consisting of 0.5 ml of euglobulin solution prepared from human serum by the method described by KLINE[1] and NORMAN[2], and 0.4 ml of $^1/_{50}$ N phosphate buffer of pH 7.2 in physiological saline (PBS) containing a test material and 0.1 ml of streptokinase solution (200 units/0.1 ml of PBS), was incubated at 37°C for 3 minutes and then 2.0 ml of fibrinogen solution (2.0 % in PBS) was added and incubated at 37°C for 20 minutes; 1.5 ml of 3.5 M perchloric acid was then added. After one hour at room temperature, the tube was centrifuged and the absorbance of the supernatant at 280 mμ was determined. The percent inhibition was calculated as follows: $a/b \times 100$, wherein a was obtained by subtraction of the absorbance with leupeptin from the absorbance without leupeptin and b was the absorbance without leupeptin.

Production and isolation of leupeptins were studied most in detail with the strain MA 839-A1. It was cultured in shake-flasks in a medium containing 1.0 % glucose, 1.0 % starch, 2.0 % peptone, 0.5 % NaCl (pH 7.0) at 27~29°C. Leupeptins were produced already after 24 hours of cultivation and the production reached a maximum after 48~72 hours. Another medium containing 2.0 % glucose, 1.0 % starch, 2.0 % peptone, 0.5 % NaCl, and 0.2 % KH_2PO_4 was employed for production in a stainless steel fermentor of 2,000 liters volume and the production reached the maximum after 40~50 hours under aeration and stirring at 27°C. The culture filtrate generally showed 50 % inhibition of plasmin activity when 0.01~0.03 ml was added to the reaction mixture in testing the antiplasmin activity. If the antiplasmin activity is assumed to be solely due to acetyl-L-leucyl-L-leucyl-DL-argininal, which was designated leupeptin Ac-LL, the amount of leupeptins in the culture filtrate was estimated to be 300~1,000 mcg/ml.

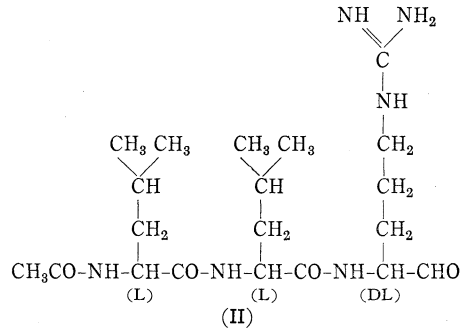
Leupeptins are adsorbed by active carbon and eluted with methanol at pH 2.0. They were more efficiently extracted, however, by ion-exchange resin processes: the culture filtrate is passed through a column of Lewatit CNP (porous carboxylic acid resin) at pH 6.0 with NaOH; leupeptins adsorbed are eluted with 1 N HCl in 80 % methanol; the active eluate is evaporated under vacuum to a brownish powder which is extracted

Fig. 1. Thin-layer chromatography using Silica Gel G
Solvent: BuOH - BuOAc - AcOH - H_2O (4 : 2 : 1 : 1)
Reagent: RYDON-SMITH or SAKAGUCHI





(I)



(II)

with 1-butanol; the butanol extract is evaporated under vacuum; the crude powder is dissolved in water and decolorized by column chromatography using Dowex 1×2 in Cl form, developing with distilled water for the fractionation; the active fractions are lyophilized. The product thus obtained showed 50 % inhibition in the antiplasmin test at about 10 mcg/ml. Separation of the acetyl forms of leupeptins from the propionyl forms was successful after leupeptins were converted to their di-*n*-butyl acetals. These were prepared by refluxing leupeptins in 1-butanol for 3 hours. The acetyl forms, which were designated di-*n*-butyl acetal of leupeptin Ac, and the propionyl forms, which were designated di-*n*-butyl acetal of leupeptin Pr, were separated by silicic acid chro-

Table 1. Effect of leupeptins and other agents on enzyme systems

Mechanism	Enzyme	Substrate	ID_{50} (μg/ml)				
			Leupeptins	ε-ACA[5]	*t*-AMCHA[6]	Try-Inh.	Trasylol[7]
Blood Coagulation	Thrombokinase	Plasma	15	> 500	> 500	19.2	> 500
	Thrombin	TAME[1]	10,000	>10,000	>10,000	>1,000	> 200
		BAEE[2]	12,000	>10,000	>10,000	>1,000	>1,000
Fibrinolysis	Plasmin	Fibrinogen	8	1,000	170	4	3
		Fibrin	6	500	100	5.7	3
		Casein	16	2,250	500	5	10
		TAME	85	4,500	1,000	80	15
Kinin Formation	Trypsin	Casein	2	> 1,000	> 1,000	0.5	2.5
		Hemoglobin	3.6	> 500	> 500	2	—
		BAA[3]	0.1	> 200	> 200	1.8	5
		TAME	65	>10,000	>10,000	2	20
		BAEE	80	>10,000	>10,000	2.5	30
	Papain	Casein	0.5	> 5,000	> 1,000	>1,000	54
		Hemoglobin	0.15	> 500	> 500	> 500	> 500
		BAA	0.05	> 200	> 200	>1,000	84
	Kallikrein	BAEE	75	>10,000	>10,000	>1,000	6
	α-Chymotrypsin	Casein	> 500	> 5,000	> 2,500	100	5.5
		ATEE[4]	>2,500	>20,000	>10,000	350	50

(1) *p*-Toluenesulfonyl-L-arginine methyl ester·HCl
(2) α-N-Benzoyl-L-arginine methyl ester·HCl
(3) α-N-Benzoyl-L-arginine amide·HCl
(4) N-Acetyl-L-tyrosine ethyl ester
(5) ε-Aminocaproic acid
(6) *trans*-4-Aminomethylcyclohexanecarboxylic acid
(7) KIU/ml. 1KIU=3 μg (by FOLIN)

matography, using butanol – butyl acetate – acetic acid – water (4 : 8 : 1 : 1 in volume) as the solvent. Di-*n*-butyl acetals of these leupeptins were converted to the leupeptins by heating at 60°C in 0.01 N HCl for 3 hours, to yield leupeptin Ac or leupeptin Pr.

The most purified leupeptin Pr hydrochloride was shown to contain mainly propionyl-L-leucyl-L-leucyl-DL-argininal (leupeptin Pr-LL) (I) by the result (leucine 1.00, isoleucine 0.10, valine 0) of the amino acid analysis of the acid hydrolysate. It showed the following properties: white powder, m.p. 110~140°C, calcd. for $C_{21}H_{40}N_6O_4 \cdot HCl \cdot H_2O$: C 50.95, H 8.76, N 16.98, O 16.16, Cl 7.16; found: C 51.13, H 8.77, N 17.10, O 14.96, Cl 5.73, $[\alpha]_D^{22}$ −56° (*c* 1, methanol); pKa′ >12; soluble in water, methanol, ethanol, butanol, acetic acid, dimethylformamide, dimethylsulfoxide; insoluble in ethyl acetate, acetone, chloroform, *n*-hexane; positive in RYDON-SMITH, SAKAGUCHI, pentacyanoaquoferriate, red tetrazolium, 2,4-dinitrophenylhydrazine; negative in ninhydrin, JAFFE, ferric chloride, anthrone, MOLISCH, benzidine; no maximum at 210~400 mμ with the end absorption.

The hydrochloride of leupeptin Ac was shown to contain mainly acetyl-L-leucyl-L-leucyl-DL-argininal (leupeptin Ac-LL) (II) by the result of the amino acid analysis (leucine 1.00, isoleucine 0.04, valine 0.01). It showed the following properties: white powder; m. p. 75~110°C; calcd. for $C_{20}H_{38}N_6O_4 \cdot HCl \cdot H_2O$: C 49.93, H 8.59, N 17.47, O 16.63, Cl 7.37; found: C 50.34, H 8.57, N 16.03, O 16.42, Cl 5.27; $[\alpha]_D^{22}$ −52° (*c* 1, methanol); pKa′ >12; the same solubilities and the same color reactions as leupeptin Pr-LL. On high-voltage paper electrophoresis (3,500 V, 15 minutes, formic acid – acetic acid – water in 25 : 75 : 900), these leupeptins behaved similarly, moving to the cathode and showing Rm 0.7~0.8 (taking L-alanine as 1.0). Leupeptins gave di-*n*-butyl acetals as described above, their reduction with the sodium borohydride gave dihydroleupeptins, and their oxidation with permanganate gave leupeptin acids. Amino acid analysis of the acid hydrolysate of leupeptin acids prepared from leupeptins Ac-LL and Pr-LL showed arginine and leucine in a molar ratio of 1 : 2. Thin-layer chromatograms of these derivatives and leupeptins are shown in the Fig. 1. Leupeptin Pr-LL or Ac-LL always shows two spots. The structures of leupeptin Ac-LL and Pr-LL were determined by the degradation studies and finally by their chemical synthesis, as reported in other papers.

As published in another paper, the biological activities of a mixture of leupeptins which showed antiplasmin activity (50 % inhibition) at 8 mcg/ml have been studied in detail. It exhibits antiplasmin activity and inhibition of trypsin, papain, and kallikrein but not chymotrypsin. It inhibits coagulation of rabbit and human bloods. The inhibition against coagulation of bloods of mice, rats and dogs are very weak. These activities of the mixture of leupeptins were compared with Trasylol, trypsin inhibitor (from soybean, Type 1-S, Sigma Chemical Co.), tranexamic acid (*trans*-4-aminomethylcyclohexanecarboxylic acid), and ε-aminocaproic acid, with the results summarized in Table 1. The type of inhibition was confirmed to be competitive with the substrate in the hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester by trypsin and in that of hemoglobin by papain. Leupeptins also showed inhibition against carrageenin inflammation in rats.

Leupeptins are well absorbed through oral route. When 1,000 mg/kg was orally given to rabbits, the highest blood level of about 200 mcg/ml was observed in serum after 1.5 hours and 2,400 mcg/ml in urine after 3~5 hours. Totally about 40 % of leupeptins administered was recovered in urine. LD_{50} was as follows: in mice, 118 mg/kg by intravenous injection, 1,405 mg/kg by subcutaneous injection, 1,550 mg/kg orally; in rats, 125 mg/kg by intravenous administration, >4,000 mg/kg by subcutaneous, >4,000 mg/kg by oral; in rabbits, 35 mg/kg by intravenous administration, 300 mg/kg by subcutaneous, and >1,500 mg/kg by oral.

Acknowledgement

The arthors are grateful to Banyu Co. for their collaboration to produce leupeptins.

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(Received February 18, 1969)

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