BIOLOGICAL ACTIVITIES OF LEUPEPTINS

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Leupeptins, leupeptin Pr and leupeptin Ac, strongly inhibit proteolysis by plasmin, trypsin and papain, but do not inhibit proteolysis by α-chymotrypsin. The inhibition is competitive with substrates. The inhibitory effect on esterolysis by plasmin and trypsin is weaker than on proteolysis. The results with derivatives of leupeptins which contain carboxyl or alcohol instead of aldehyde and of di-*n*-butyl acetals of leupeptins indicate that the free aldehyde group plays a role in the activities. Leupeptins are absorbed orally and at least about 25 % is excreted in urine. Oral administration of leupeptins exhibited an anti-inflammatory effect on edema. Leupeptins inhibited thrombokinase reaction and coagulation of blood of human and rabbit. Type of inhibition was different from heparin. Coagulation of blood of rats and dogs are not inhibited. The effects of leupeptins on thrombokinase, thrombin, plasmin, trypsin, papain, kallikrein and α-chymotrypsin were compared with those of ε-aminocaproic acid, *trans*-4-aminomethylcyclohexanecarboxylic acid, soybean trypsin inhibitor and trasylol.

Leupeptins are acetyl- or propionyl-L-leucyl-L-leucyl-DL-argininals and their analogues in which L-leucine is replaced with L-valine or L-isoleucine. They are produced by various species of actinomycetes[1,2,3]. The former was designated leupeptin-Ac and the latter leupeptin-Pr. As briefly reported previously[1], they inhibit effects of plasmin, trypsin, papain, thrombokinase and coagulation of human and rabbit blood. In this paper, these activities of leupeptins and their derivatives are reported in detail.

Materials and Methods

Human plasminogen: Euglobulin fraction containing plasminogen was prepared from human serum by the method described by KLINE[4] and NORMAN[5]. It was dissolved in phosphate buffer saline (PBS, 0.02 M, pH 7.2) of the same volume of the original serum and centrifuged for 30 minutes at 10,000 g and the supernatant containing plasminogen was employed for all experiments.

Streptokinase: A commercial product (Varidase) by Lederle Laboratories, U. S. A. was employed. A stock solution containing 10,000 units per ml in PBS was prepared and stored in a refrigerator.

Thrombin: A commercial product produced by Mochida Pharmaceutical Co., Ltd., Japan was employed.

Trypsin: Trypsin (1 : 250) purchased from Difco Co., U.S.A. was employed. It was dissolved and diluted with PBS, 0.01 M Tris buffer of pH 8.2 or 0.01 M borate buffer of pH 7.4 immediately before use.

α-Chymotrypsin: The product purchased from Sigma Co., U. S. A. was employed. It was dissolved and diluted with PBS or borate buffer immediately before use.

Papain: Papain (2X. cryst.) purchased from Sigma Co., U. S. A. was employed. It was dissolved and diluted with PBS or borate buffer immediately before use.

Kallikrein: The product of Bayer Co., Germany, for medical use containing 10 biological units in an ampoule was employed. It was dissolved and diluted with Tris buffer immediately before use.

Thrombokinase and ox plasma: Thrombokinase was prepared from acetone powder of ox brain. Two hundred mg of acetone powder was heated with 5 ml of physiological saline at 50°C for 5 minutes, and after cooling, centrifuged at 1,500 g for 5 minutes. The supernatant which was designated thrombokinase solution was employed for experiments. Ox plasma was prepared by addition of 20 ml of 10 % Na_2SO_4 to 100 ml of ox blood and centrifugation at 3,000 g thereafter.

Fibrinogen: Bovine fibrinogen 1 purchased from Armour Pharm. Co., U. S. A. was employed.

Casein: Purified casein was prepared according to the procedure described by NORMAN[5]. The purity was as follows: adding 2.0 ml of 1.7 M perchloric acid to 2.0 ml of 2.0 % casein solution, the optical absorbance at 280 mμ of the supernatant was less than 0.1.

Hemoglobin: Bovine hemoglobin for enzyme substrate (MW about 68,000) purchased from Nutritional Biochem. Co., U.S.A. was employed.

Synthetic substrates: *p*-Toluenesulfonyl-L-arginine methyl ester hydrochloride (TAME) and N-acetyl-L-tyrosine ethyl ester (ATEE) were purchased from Tokyo Kasei Kogyo Co., Japan. α-N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) and α-N-benzoyl-L-arginine amide hydrochloride (BAA) were purchased from Sigma Co., U. S. A.

Inhibitors: The mixture of leupeptins consisting of leupeptin Pr and leupeptin Ac (1 : 3), and each of these leupeptins were employed. The processes for preparation of these leupeptins are reported in other papers[2,3]. ε-Aminocaproic acid (ε-ACA) and *trans*-4-aminomethylcyclohexanecarboxylic acid (*t*-AMCHA) purchased from Daiichi Seiyaku Co., Japan were employed. Trasylol, a product of Bayer Co., Germany for medical use containing 25,000 KIU/5 ml in an ampoule and soybean trypsin inhibitor (3X cryst.) purchased from Sigma Co., U.S.A. were employed.

Method of determination of proteolytic reactions: After the reactions described below, increase in extinction of a reaction mixture was read at 280 mμ, at 750 mμ after LOWRY reaction[6] or at 570 mμ after ninhydrin reaction[7].

Method of determination of esterolytic reactions: After the reactions described below, amount of esters such as TAME, BAEE and ATEE which remained after the reaction was determined by hydroxylamine-ferric chloride method, measuring the extinction at 525 mμ[8].

Estimation of percent inhibition on proteolytic reactions and esterolytic reactions: The percent inhibition of proteolytic reactions was calculated as follows:

$$\% \text{ Inhibition} = \frac{A - B}{A} \times 100$$

A = Activity without inhibitor
B = Activity with inhibitor

The percent inhibition on esterolytic reactions was calculated as follows:

$$\% \text{ Inhibition} = \frac{(A-B)-(A-C)}{(A-B)} \times 100$$

A = Activity without inhibitor and enzyme
B = Activity without inhibitor
C = Activity with inhibitor

From the inhibition curve taking probit of inhibition percent at varied concentrations of an inhibitor on ordinate and logarithm of concentrations of an inhibitor on abscissa, the concentration for 50 % inhibition was obtained. In all cases, a linear relation was observed between 20 % and 80 % inhibition.

Buffers: Unless otherwise specified, buffers employed were as follows: PBS in this paper contains $Na_2HPO_4 \cdot 12H_2O$ 2.9 g, KH_2PO_4 0.2 g, NaCl 8 g, KCl 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.1 g and $MgCl_2 \cdot 6H_2O$ 0.1 g per 1 liter at pH 7.2; Tris-HCl buffer is 0.01 M of pH 8.2; Borate buffer is 0.01 M of pH 7.4.

Reaction mixture for fibrinolysis by plasmin: Five tenth ml of plasminogen solution, 0.1 ml of PBS, 0.1 ml of thrombin solution (50 units/ml) and 0.2 ml of PBS with or without an inhibitor were mixed and incubated at 37°C for 3 minutes, and 0.1 ml of streptokinase solution (2,000 units/ml) was added and kept at 37°C for 5 minutes. Thereafter, 2.0 ml of 2.0 % fibrinogen solution was added, and the reaction mixture was incubated at 37°C for 30 minutes. Then, 2.0 ml of 1.7 M perchloric acid was added, after 1 hour at room temperature the reaction mixture was centrifuged, and the extinction of the supernatant was read at 280 mμ.

Reaction mixture for fibrinogenolysis by plasmin: The reaction mixture was same as that for fibrinolysis by plasmin except that instead of thrombin solution, 0.1 ml of PBS was added.

Reaction mixture for hydrolysis of casein by plasmin: Five tenth ml of plasminogen solution 1.0 ml of 4 % casein solution and 0.2 ml of borate buffer with or without inhibitor were mixed and incubated for 3 minutes and 0.1 ml of streptokinase solution (2,000 units/ml) was added. It was incubated at 37°C for 20 minutes, 2.0 ml of 1.7 M perchloric acid was added, after 1 hour at room temperature, it was centrifuged and the extinction was read at 280 mμ.

Reaction mixture for hydrolysis of casein by trypsin, papain or α-chymotrypsin: One ml of 2.0 % casein solution, 0.5 ml of borate buffer and 0.3 ml of borate buffer with or without inhibitor were mixed and incubated for 3 minutes. Then, 0.2 ml of a solution of trypsin (100 μg/ml), papain (500 μg/ml) or α-chymotrypsin (50 μg/ml, containing 0.05 ml of 0.02 M $CaCl_2$) was added. It was incubated at 37°C for 30 minutes. Then, 2.0 ml of 1.7 M perchloric acid was added, after 1 hour it was centrifuged and the extinction was read at 280 mμ.

Reaction mixture for proteolysis of BAA by trypsin: Two tenth ml of 0.01 M BAA, 0.5 ml of PBS, and 0.2 ml of PBS with or without inhibitor were mixed and incubated at 37°C for 3 minutes, and 0.1 ml of trypsin (1.0 mg/ml) was added. After incubation at 37°C for 20 minutes, 0.5 ml of cyanide-acetate (2.0 ml of 0.01 M NaCN mixed with 98 ml of acetate buffer of pH 5.3) and 0.5 ml of 3.0 % ninhydrin solution were added, after 15 minutes boiling, it was diluted with 5 ml of isopropanol and the extinction was read at 570 mμ[7].

Reaction mixture for esterolysis of TAME by plasmin: Two tenth ml of 0.1 M TAME and 0.55 ml of 0.02 M Tris buffer with or without inhibitor were mixed and incubated for 3 minutes and 0.25 ml of plasmin solution activated with streptokinase were mixed. After incubation at 37°C for 20 minutes, 2.0 ml of alkaline hydroxylamine solution (2 M hydroxylamine·HCl mixed with equal volume of 3.5 M NaOH) was added, and after 25 minutes at room temperature, 1.0 ml of 12 % hydrochloric acid solution containing trichloracetic acid at 6 % was added. One ml of this solution was added to 4.0 ml of ferric chloride (0.11 M) in 0.04 N HCl and the extinction was read at 525 mμ. The plasmin solution was prepared as follows: euglobulin fraction was dissolved in Tris buffer of three quaters volume of the original human serum, then it was mixed with streptokinase solution (10,000 units/ml) in a 3:2 volume ratio and kept for 3 minutes at 37°C.

Reaction mixture for esterolysis of TAME by trypsin or thrombin: The reaction

mixture was same as that for esterolysis by plasmin except that instead of plasmin solution, 0.25 ml of thrombin solution (10 units/ml) or trypsin solution (100 μg/ml) was added.

Reaction mixture for esterolysis of BAEE by thrombin, trypsin or kallikrein: One tenth ml of 0.1 M BAEE and 0.8 ml of Tris buffer with or without inhibitor were mixed and incubated for 3 minutes, and 0.1 ml of thrombin solution (500 units/ml), trypsin (750 μg/ml) or kallikrein (5 units/ml) was added. The reaction mixture was incubated for 20 minutes at 37°C, and after the reaction it was treated as in the case of the esterolysis by plasmin and the extinction was read at 525 mμ.

Reaction mixture for esterolysis of ATEE by α-chymotrypsin: Five tenth ml of 0.04 M ATEE and 0.25 ml of PBS with or without inhibitor were mixed and incubated for 3 minutes, and 0.25 ml of α-chymotrypsin (10 μg/ml) was added. After incubation at 37°C for 20 minutes the reaction mixture was treated as in the case of esterolysis by plasmin and extinction was read at 525 mμ.

Inhibition of thrombokinase: One tenth ml of thrombokinase solution, 0.3 ml of saline and 0.1 ml of saline with or without inhibitor were mixed, and 1.0 ml of ox plasma was added. Time necessary for the coagulation was determined, and the concentration to prolong the coagulation time to twice longer than that of the control was obtained from the curve taking the coagulation time on ordinate and concentrations of the inhibitor on absissa.

Method of testing the effect of leupeptins on blood coagulation: A mixture containing leupeptin Pr and leupeptin Ac in the ratio of 1 : 3 was employed. A half ml of the leupeptins solution diluted with saline was placed in each test tube. Then, 2.0 ml of blood of human, rabbit or rat was added and the occurrence of the coagulation was examined 5, 10, 20, 30, 40, 50 and 60 minutes thereafter: $-$ means no coagulation, $\pm$ a slight coagulation, $+$ complete coagulation, $++$ complete coagulation with shrinking of the clot and appearance of serum.

Method of testing the effect on carrageenin inflammation: Carrageenin was purchased from Banyu Seiyaku Co., Japan. It was dissolved in saline at 1.0 %. The leupeptins or α-chymotrypsin dissolved in saline was orally given to rats and 1 hour thereafter, 0.05 ml of the carrageenin solution was injected subcutaneously to the foot pad. Thirty minutes, 1, 2, 3 and 4 hours thereafter the extent of edema measured by the methed described by WINTER *et al.*[9]

Method of testing the concentrations of leupeptins in serum and urine: The activity of leupeptins in serum and urine was determined by assaying fibrinogenolysis by plasmin.

Results and Discussion

As reported in a previous paper[3], leupeptin Pr and leupeptin Ac form di-*n*-butyl acetals[2]. These di-*n*-butyl acetals and leupeptins were compared for their inhibitory effects on reactions of thrombokinase, thrombin, plasmin, trypsin, papain, kallikrein and α-chymotrypsin. The results are summarized in Table 1. In this table, the concentrations for 50 % inhibition are indicated. The leupeptins strongly inhibit thrombokinase reaction, fibrinogenolysis and fibrinolysis by plasmin, proteolysis of casein, hemoglobin and BAA by trypsin, proteolysis of casein, hemoglobin and BAA by papain. They show weaker inhibition against esterolysis of TAME by plasmin, TAME and BAEE by trypsin, and BAEE by kallikrein. The leupeptins have no or almost no activity to inhibit esterolysis of TAME and BAEE by thrombin and proteolysis by α-chymotrypsin. These results indicate that the leupeptins inhibit more strongly the proteolytic effects of plasmin, trypsin and papain than their esterolytic effects, but the leupeptins do not inhibit the activity of α-chymotrypsin. Fibrino-

Table 1. Effect of leupeptins and these derivatives on enzyme systems

Enzymes	Substrates	ID$_{50}$ (μg/ml)			
		Pr acetal *5)	Ac acetal *6)	Pr *7)	Ac *8)
Thrombokinase	Plasma	>500	>500	15	18
Thrombin	TAME *1)	>500	>500	10,000	12,000
	BAEE *2)	>500	>500	12,000	15,000
Plasmin	Fibrinogen	>500	>500	8	10
	Fibrin	>500	>500	6	8
	Casein	>500	>500	14	36
	TAME	>500	>500	67	100
Trypsin	Casein	>500	>500	2	2
	Hemoglobin	265	705	4.3	5
	BAA *3)	3.5	38.4	0.1	0.1
	TAME	>500	>500	93	93
	BAEE	>500	>500	136	113
Papain	Casein	133	150	0.48	0.51
	Hemoglobin	3.6	107	0.15	0.15
	BAA	4.3	45.1	0.03	0.08
Kallikrein	BAEE	>500	>500	75	70
α-Chymotrypsin	Casein	>500	>500	>500	>500
	ATEE *4)	>500	>500	>500	>500

*1) *p*-Toluenesulfonyl-L-arginine methyl ester HCl *2) α-N-Benzoyl-L-arginine ethyl ester HCl
*3) α-N-Benzoyl-L-arginine amide HCl *4) N-Acetyl-L-tyrosine ethyl ester
*5) Propionyl-L-leucyl-L-leucyl-DL-argininal di-*n*-butyl acetal
*6) Acetyl-L-leucyl-L-leucyl-DL-argininal di-*n*-butyl acetal
*7) Propionyl-L-leucyl-L-leucyl-DL-argininal *8) Acetyl-L-leucyl-L-leucyl-DL-argininal

genolysis and fibrinolysis by plasmin were more strongly inhibited by leupeptins than the proteolysis of casein. Though the effect on proteolytic effect of kallikrein was not tested, the inhibition on the esterolytic activity of kallikrein suggests that the leupeptins would have strong activity against the proteolytic effect of kallikrein. Di-*n*-butyl acetals of leupeptins show no inhibition against almost all reactions which were strongly inhibited by the leupeptins except that they show fairly strong inhibition of proteolysis of BAA by trypsin, hemoglobin and BAA by papain, and weak inhibition against proteolysis of hemoglobin by trypsin and casein by papain. Leupeptin Pr has a slightly stronger activity than leupeptin Ac against all systems inhibited. The di-*n*-butyl acetal of leupeptin Pr shows much stronger activity against a few systems than the di-*n*-butyl acetal of leupeptin Ac.

The differences of the activities between the leupeptins and their di-*n*-butyl acetals suggest an important role of aldehyde group for the activity. It was further confirmed by testing the effect of leupeptin derivatives in which the aldehyde group is oxidized to carboxyl or reduced to alcohol. As shown in Table 2, these derivatives exhibit no effect against fibrinogenolysis by plasmin and proteolysis of casein by papain.

Kinetic studies have been done on the effect of leupeptin Pr and leupeptin Ac on esterolysis of TAME by trypsin and on proteolysis of hemoglobin by papain. Typical

results are shown on a Lineweaver-Burk plot in Fig. 1 (a, b). The inhibition is of the competitive type. Km and Ki were as follows: Km for trypsin or papain of these respective substrates was 4.8×10^{-3}M and 7.1×10^{-5}M, respectively. Ki of leupeptin Pr or leupeptin Ac for esterolysis of TAME by trypsin was 4.4×10^{-5}M and 8.1×10^{-5}M. Ki of leupeptin Pr or leupeptin Ac for proteolysis of hemoglobin by papain was 1.3×10^{-6}M and 1.4×10^{-6}M, respectively.

The effect of the mixture of leupeptins consisting of leupeptin Pr and leupeptin Ac in the ratio of 1 : 3 were compared with ε-ACA, *t*-AMCHA, soybean trypsin inhibitor and trasylol, and the results are summarized in Fig. 2 and Table 3. Fig. 2 shows graphical determination of their ID_{50}. Leupeptins were found to be much more potent inhibitors against fibrinogenolysis, fibrinolysis and caseinolysis by plasmin than ε-ACA and *t*-AMCHA. Leupeptins, soybean trypsin inhibitor and trasylol exhibit strong inhibition of plasmin. Soybean trypsin inhibitor does not show strong inhibition of papain but inhibits α-chymotrypsin weakly.

Table 2. Relationship between chemical structures and biological activities

Structure of Leupeptins

$R_1-NH-CH(CH_2CH(CH_3)_2)-C(=O)-NH-CH(CH_2CH(CH_3)_2)-C(=O)-NH-CH(CH_2CH_2CH_2-NH-C(=NH)NH_2)-R_2$

No.	R_1	R_2	Activity (ID_{50} μg/ml)	
			Plasmin *1)	Papain *2)
I	CH_3CH_2CO-	-CHO	8	0.48
II	CH_3CO-		10	0.51
III	CH_3CH_2CO-	-COOH	>500	>500
IV	CH_3CO-		>500	>500
V	CH_3CH_2CO-	$-CH_2OH$	>500	>500
VI	CH_3CO-		>500	>500
VII	CH_3CH_2CO-	$CH(OCH_2CH_2CH_2CH_3)_2$	>500	133
VIII	CH_3CO-		>500	150

I) Propionyl-L-leucyl-L-leucyl-DL-argininal
II) Acetyl-L-leucyl-L-leucyl-DL-argininal
III) Propionyl-L-leucyl-L-leucyl-DL-arginine
IV) Aectyl-L-leucyl-L-leucyl-DL-arginine
V) Propionyl-L-leucyl-L-leucyl-DL-argininol
VI) Acetyl-L-leucyl-L-leucyl-DL-argininol
VII) Propionyl-L-leucyl-L-leucyl-DL-argininal di-*n*-butyl acetal
VIII) Acetyl-L-leucyl-L-leucyl-DL-argininal di-*n*-butyl acetal

*1) Fibrinogenolysis by plasmin, *2) Caseinolysis by papain

Fig. 1.

a) Kinetics of inhibition by leupeptin Pr of papain-hemoglobin system.

b) Kinetics of inhibition by leupeptin Pr of trypsin-TAME system.

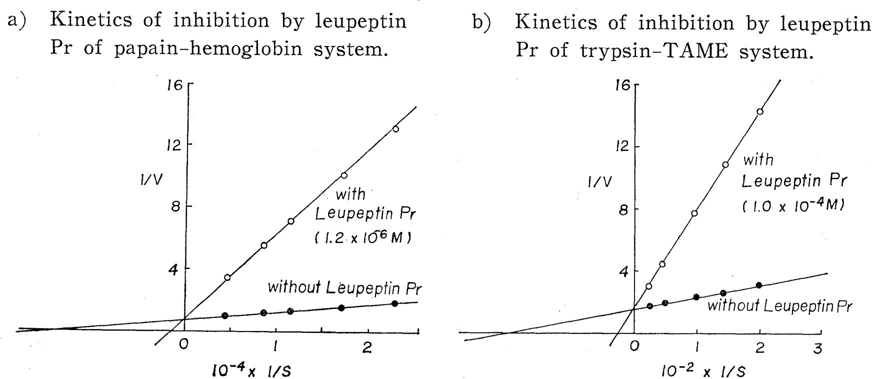


Table 3. Effects of leupeptins, ε-ACA, *t*-AMCHA, soybean trypsin inhibitor and trasylol on enzyme systems

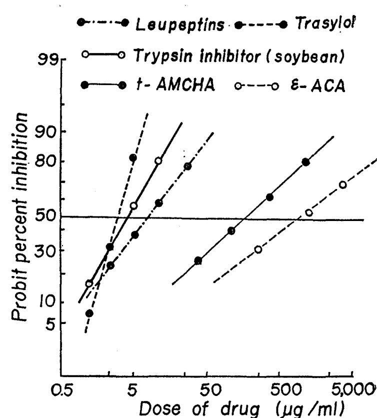
Enzymes	Substrates	Leupeptins	ID50 (μg/ml)			
			ε-ACA *5)	*t*-AMCHA *6)	Try-Inh.	Trasylol *7)
Thrombokinase	Plasma	15	>500	>500	19.2	>500
Thrombin	TAME *1)	10,000	>10,000	>10,000	>1,000	>200
	BAEE *2)	12,000	>10,000	>10,000	>1,000	>1,000
Plasmin	Fibrinogen	8	1,000	170	4	3
	Fibrin	6	500	100	5.7	3
	Casein	16	2,250	500	5	10
	TAME	85	4,500	1,000	80	15
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 ethyl 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. 1KIU=3 μg (by Folin)

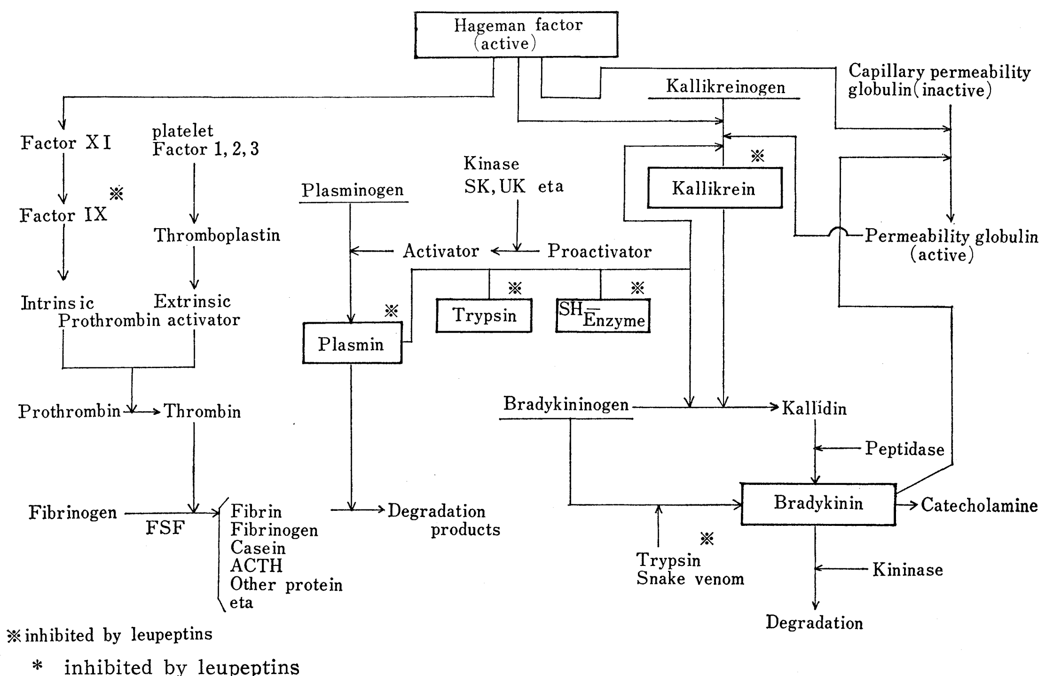
Trasylol, unlike the leupeptins, does not inhibit thrombokinase, but inhibits α-chymotrypsin strongly and papain very weakly.

Leupeptins which inhibit thrombokinase inhibit blood coagulation. This effect was studied in comparison with heparin. It is interesting that leupeptins show strong inhibition of coagulation of human and rabbit blood but do not inhibit the coagulation of mouse, rat and dog blood. The results with rabbit blood is shown in Table 4. The type of inhibition by leupeptins is different from heparin: no coagulation occurred in the system containing heparin at 4 μg/ml after 5 minutes, and no coagulation occurred in the system containing heparin at 15.6 μg/ml even after 60 minutes; no coagulation occurred in the system containing the mixture of leupeptins at 2 μg/ml after 5 minutes, no coagulation occurred at 31.3 μg/ml of leupeptins after 10 minutes, but after 60 minutes coagulation occurred even at 1,000 μg/ml of leupeptins. With human blood, inhibition of coagulation was observed at 10 μg/ml of the mixture of leupeptins

Fig. 2. Effects of leupeptins, try-inh., trasylol, *t*-AMCHA and ε-ACA on fibrinogenolysis by plasmin.

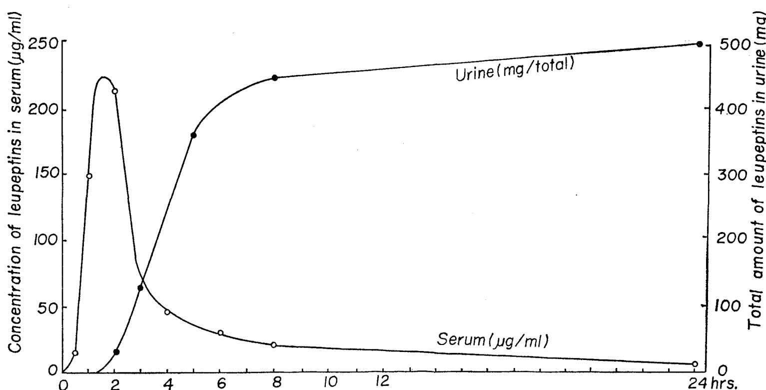


Scheme 1. Mode of inhibition of leupeptins on blood coagulation, fibrinolysis and kinin formation



* inhibited by leupeptins

Fig. 3. Serum and urine concentrations of leupeptins in rabbit by oral administration.



after 10 minutes, and after 60 minutes coagulation occurred at 50 μg/ml. After 60 minutes, the lowest concentration to inhibit coagulation of human blood was 400 μg/ml. The inhibition of leupeptins of the enzyme systems of blood coagulation, fibrinolysis and kinin formation mechanisms are shown in Scheme 1.

The toxicity of leupeptins by intravenous or subcutaneous injection may be related to inhibition of blood coagulation. One thousand μg/ml of the mixture of leupeptins showed no inhibition of coagulation of blood of rats and mice. LD_{50} to rats and rabbits were as follows: rats 125 mg/kg (75~150 mg/kg) by intravenous injection, larger than 4,000 mg/kg by subcutaneous injection and oral administration; rabbits 35 mg/kg

(25~50 mg/kg) by intravenous injection, 300 mg/kg (200~400 mg/kg) by subcutaneous injections, larger than 1,500 mg/kg by oral administration.

Leupeptins are absorbed through the oral route. One thousand mg/kg of the mixture of leupeptins was given to rabbits weighing 2.0~2.5 kg, and 30 minutes, 1, 2, 3, 4, 6, 8 and 24 hours thereafter, the concentrations in the serum were determined by testing the inhibition on fibrinogenolysis by plasmin. As shown in Fig. 3, at two hours after the oral administration about 200 μg/ml of leupeptins was found in serum. The urine excreted during 0~2, 2~3, 3~5, 5~8 and 8~24 hours after the administration was collected and the amount of excreted leupeptins were determined. In Fig. 3, the total amount excreted at various times after administration is shown. Totally about 500 mg was excreted in urine and most of it was excreted within 5 hours after the administration. The amount excreted suggests that at least 25 % of that orally administrated is absorbed.

Table 4. Effect of leupeptins and heparin on coagulation time of rabbit blood

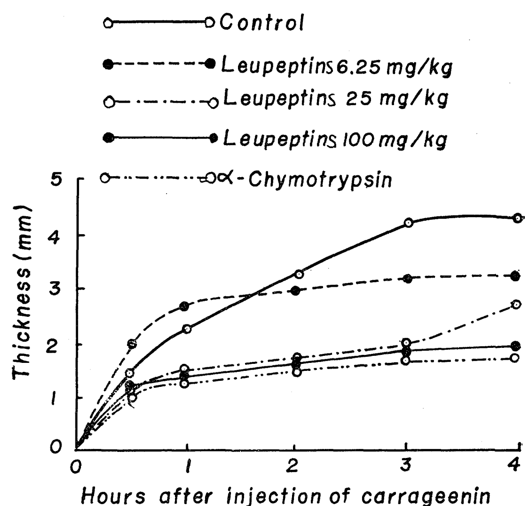
Conc. of leupeptins (μg/ml)	Intensity of blood coagulation (min.)				Conc. of heparin (μg/ml)	Intensity of blood coagulation (min.)			
	5	10	30	60		5	10	30	60
1,000	− −	− −	− −	+ +					
500	− −	− −	− −	+ +	500	− −	− −	− −	− −
250	− −	− −	− −	+ ++	250	− −	− −	− −	− −
125	− −	− −	− +	+ ++	125	− −	− −	− −	− −
62.5	− −	− −	+ +	++ ++	62.5	− −	− −	− −	− −
31.3	− −	− −	+ +	++ ++	31.3	− −	− −	− −	− −
15.6	− −	± −	++ +	++ ++	15.6	− −	− −	− −	− −
8	− −	± +	++ ++	++ ++	8	− −	− −	± +	+ +
4	− −	± +	++ ++	++ ++	4	− −	+ +	+ +	+ +
2	− −	+ +	++ ++	++ ++	2	+ +	+ +	+ +	+ +
1	± ±	+ +	++ ++	++ ++	1	+ +	+ +	+ +	++ ++
0.5	+ +	+ +	++ ++	++ ++					
0.25	+ +	+ +	++ ++	++ ++					
0.125	+ +	+ ++	++ ++	++ ++					
Control	+ +	+ ++	++ ++	++ ++					

The oral administration of leupeptins also exhibits anti-inflammatory effect. The results from the experiment testing the effect of the mixture of leupeptins are shown in Fig. 4. For comparison, α-chymotrypsin was tested. The oral administration of 100 mg/kg of leupeptins and the same dose of α-chymotrypsin administered intraperitoneally showed the similar strength of inhibition of carrageenin edema. Weak inhibition was observed with oral administration of 25.0 mg/kg or 6.25 mg/kg of leupeptins.

LEWIS[10], BACK[11] and HENRIQUES[12] and others reported that plasmin played an important role in conversion of kallikreinogen to kallikrein and kininogen to kinin. Trypsin and cathepsin were demonstrated to take part in for mation of kinin by GREENBAUM[13]. On the other hand, UDAKA and HAYASHI *et al.*[14,15,16] reported that SH enzymes such as protease of Arthus inflammation and protease of heat inflamma tion played an important role in causing inflammation. As reported in this papar,

proteolytic effects of plasmin, kallikrein and papain which are SH enzymes are inhibited by leupeptins. Leupeptins inhibit fibrinogenolysis and fibrinolysis, while they inhibit coagulation of human and rabbit blood. On the basis of these activities and these oral absorption, oral or topical applications of leupeptins should be tested against inflammatory diseases.

Fig. 4. Effect of leupeptins on carrageenin edema of rats.



Leupeptins, which can be chemically synthesized, were first discovered in culture filtrates of various species of actinomycetes. Perhaps organisms which produce a protease produce at the same time an inhibitor to protect themselves from the emzymatic effect. Leupeptins have been isolated from more than 10 species of actinomycetes, and may be of general physiological significance for actinomycetes. It is also interesting that the leupeptins produced by actinomycetes are small molecules, compared with the macromolecular trypsin inhibitor of soybean and trasylol of animal origin.

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