THE INHIBITION OF THE ENZYMIC ACTIVITY OF BLOOD COAGULATION AND FIBRINOLYTIC SERINE PROTEASES BY A NEW LEUPEPTIN-LIKE INHIBITOR, AND ITS STRUCTURAL ANALOGUES, ISOLATED FROM *STREPTOMYCES GRISEUS*

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A group of leupeptin analogues was found in *Streptomyces griseus* strain 254, isolated from a soil sample from Fujian Province, China. The inhibitors excreted in the culture filtrate were purified by adsorption on macroporous resin, followed by sequential ion exchange chromatography on DEAE-52 cellulose, CM-32 cellulose and affinity chromatography with immobilized trypsin. The preparation thus obtained was further purified by preparative HPLC. Several major components were found and characterized, which possessed different inhibitory properties toward trypsin. Based upon amino acid and mass spectrophotometric analysis, these peptides were placed in four major structural categories, *viz.*, R-Val-Val-argininal, R-Leu-Leu-argininal, R-Ile-Ile-argininal and R-Thr-Thr-argininal, this latter component representing a newly identified leupeptin analogue. The structural variability of the R-group was partly responsible for the multiplicity of the peaks obtained with HPLC. All peptides displayed varying degrees of inhibitory activity toward proteases involved in blood coagulation and fibrinolysis, including plasmin, factor Xa, activated protein C and thrombin. Among these peptide inhibitors, the molecule containing threonine showed the strongest inhibitory activity.

Leupeptin-like inhibitors have been identified in various strains of Streptomyces[1] and have been found to possess structures containing R-L-Leu-L-Leu-L-argininal (leupeptin), R-L-Val-L-Val-L-argininal and R-L-Ile-L-Ile-L-argininal, where R is propionyl or acetyl[2,3]. The mixture of acetyl- and propionyl-leupeptin has been found to inhibit enzymes, belonging to the general classes of serine proteases, thiol proteases and metallo-proteases[1,4], thus providing this inhibitor with a wide variety of effects on cellular function and *in vivo* properties[5~7].

We have found that a variety of leupeptin-like molecules exist in *Streptomyces griseus* and desired to partially characterize their structural features and ability to inhibit a variety of proteases involved in blood coagulation and fibrinolysis. We desired to develop an HPLC method for identification and mild purification of these peptides, since the normal separation of leupeptins containing different R groups relied on reflux and extraction conditions wherein racemization of the L-argininal occurred, providing a mixture in which only a portion of the molecules possessed inhibitory properties[8,9]. This report represents a summary of our efforts in this regard.

Materials and Methods

Materials

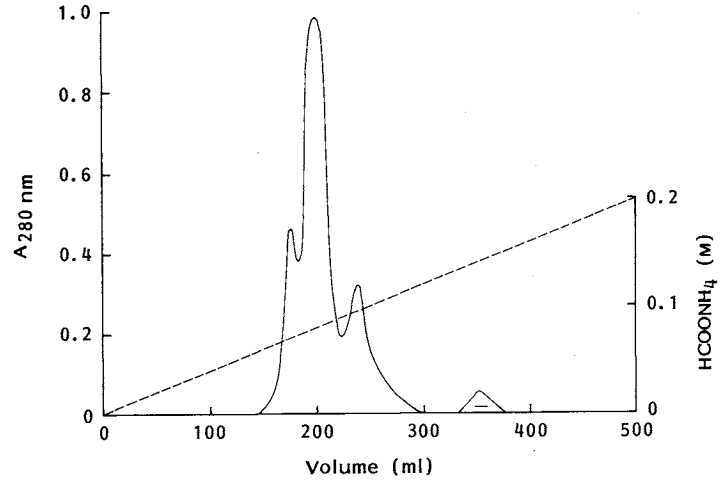
S. griseus strain 254 was isolated from a soil sample collected from Fujian Province, China. Trypsin,

thrombin and leupeptin were purchased from the Sigma Chemical Company. Bovine activated protein C[10], bovine factor Xa[11] and human plasmin[12] were prepared as described. The chromogenic substrates, S2251 (D-Val-L-Leu-L-Lys-*p*-nitroanilide), S2238 (D-Phe-L-pipecolyl-L-Arg-*p*-nitroanilide) and S2222 (Bz-D-Ile-L-Glu-Gly-L-Arg-*p*-nitroanilide) were purchased from Helena Laboratories.

Fermentation and Extraction of Leupeptin Analogues

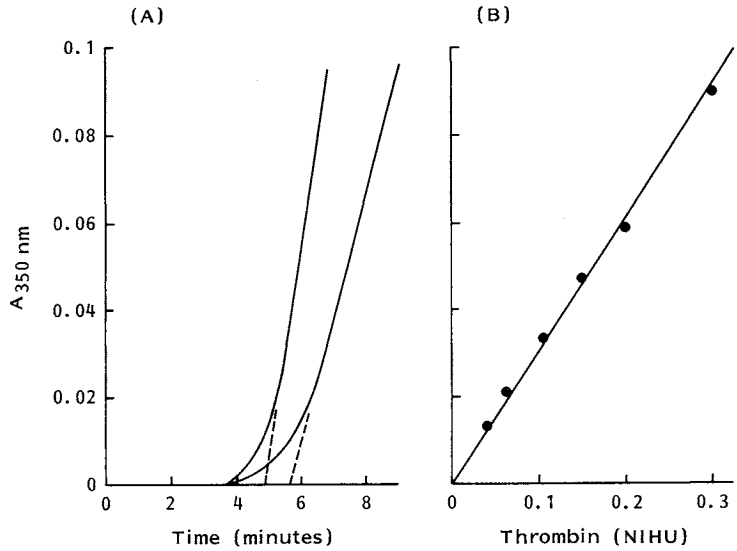
S. griseus was grown in a medium containing starch 1% (w/v), peptone 1.5% (w/v), glycerol 1%, yeast paste 0.3% (w/v) and NaCl 0.1% (w/v), pH 6.8~7.0, for 4 days at 28°C. The cultured broth (4 liters) was centrifuged and the pH of the supernatant was adjusted to 3.0 by addition of 2N HCl. The formed precipitate was removed by centrifugation and the pH of the supernatant was readjusted to pH 7.5. This

Fig. 1. Elution profile of a crude leupeptin preparation from CM-32 cellulose.



The volume of each fraction was 5 ml. All active material was found in the last small peak.

Fig. 2. The relationship between the thrombin concentration and the turbidity increment of fibrin.



(A) A total of 0.2 and 0.3 NIH units of thrombin was employed in a 1-ml reaction mixture containing 1.5 mg of fibrinogen in 0.05 M Hepes-NaOH, pH 7.4. The linear portion of the curves was extrapolated to the abscissa for the measurement of the turbidity increment of fibrin.

(B) The turbidity increments of fibrin were determined as a function of the thrombin concentrations.

material was applied to an unionized-16 macroporous resin column (3.2 × 40 cm), equilibrated with H_2O. The column was eluted with H_2O until the eluate was colorless, and then with 50% methanol-H_2O, pH 5.0. The active fractions (assayed against trypsin) were pooled and concentrated to dryness, under vacuum at 40°C. The brownish powder was dissolved in a small volume of H_2O and applied to a DEAE-52 cellulose column (3 × 28 cm), equilibrated with 0.02 M NH_4HCO_3. The column was washed with the equilibration solution. The active fractions were pooled and reconcentrated to a small volume, at 40°C, under vacuum, and subjected to chromatography on a CM-32 cellulose column (2.2 × 38 cm), equilibrated with 0.02 M ammonium formate, pH 4.0. The column was washed with this same solution until the eluate was colorless. Materials were then eluted with a linear gradient from 0.02 to 0.2 M ammonium formate, pH 4.0. The results are shown in Fig. 1. The active fractions were present in the last small peak. This pool was dried under vacuum and employed as the crude material for further purification.

Enzyme Assays

The chromogenic substrate, S2238, was used to assay trypsin and activated protein C, S2251 was employed for assay of plasmin, and S2222 for factor Xa. Assays were conducted at 37°C using a Cary 219 recording spectrophotometer. The stock buffer was 0.5 M Tris-HCl, pH 7.4. In a typical assay, a total of 0.1 ml of buffer and 0.02 ml of enzyme were placed in the cuvette. Increasing amounts of the leupeptin analogue were next added, followed by H_2O, and 0.02 ml of a fixed concentration of the desired substrate. The final assay volume was 1.0 ml. The rate of enzymic hydrolysis of the substrate was continually recorded (405 nm) and decreased with increasing concentrations of inhibitor. The experiment was then repeated at a second substrate concentration.

Thrombin activity was determined by the turbidity increment monitored at 350 nm accompanying the polymerization of fibrin monomer. After a latent time required for the thrombin-catalyzed release of fibrinopeptides A and B from fibrinogen, polymerization of fibrin occurred with an increase in turbidity, which was parallel to the thrombin concentration (Fig. 2).

Amino Acid Analysis

Samples for amino acid analysis were hydrolyzed for 20, 30 and 40 hours in 6 N HCl at 110°C in tubes sealed under vacuum. Amino acid analysis was carried out on *o*-phthaldialdehyde-precolumn derivatized samples. The HPLC apparatus consisted of a Spectra Physics HPLC, with a Model 8800 Ternary Pump, a SP4290 integrator, and a Rheodyne manual injector with a 20-μl injection loop. Fluorescence detection was accomplished with a Varian Fluorochrome flow-through filter fluorometer. The excitation and emission filters covered the ranges of 305~395 nm and 420~650 nm, respectively. An Ultrasphere ODS, 3 μm, column (4.6 × 100 mm, i.d.) was used for the separations. We have achieved optimal success with the following separation gradient, consisting of two solutions, *viz.*, 80% 0.1 M NaOAc-20% methanol-0.4% THF, pH 5.8 (solution A), and 100% methanol (solution B). The column was equilibrated with solution A. After application of the sample, the column was developed at a flow rate of 1.5 ml/minute with the following linear gradients: 90% A-10% B to 80% A-20% B for 10 minutes; 80% A-20% B to 60% A-40% B for 2.5 minutes; 60% A-40% B to 20% A-80% B for 10 minutes; and 20% A-80% B to 100% B for 0.5 minute. This procedure led to resolution of all amino acids in a standard amino acid sample.

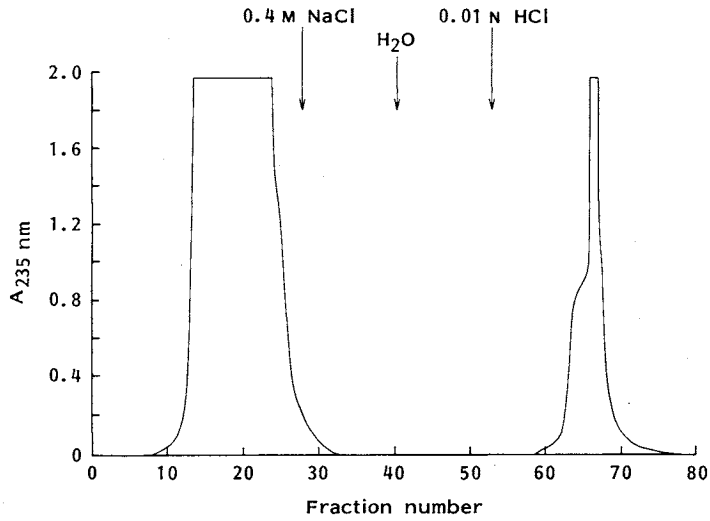
Mass Spectral Analysis

Positive and negative fast atom bombardment (FAB)-MS were obtained at approximately 8 kV, under xenon, with a Finnegan-MAT Model 8430 mass spectrometer. Prior to analysis, each sample was dissolved in H_2O, followed by addition of a drop of glycerol. In all cases the sample was scanned to 1,020 amu.

Results

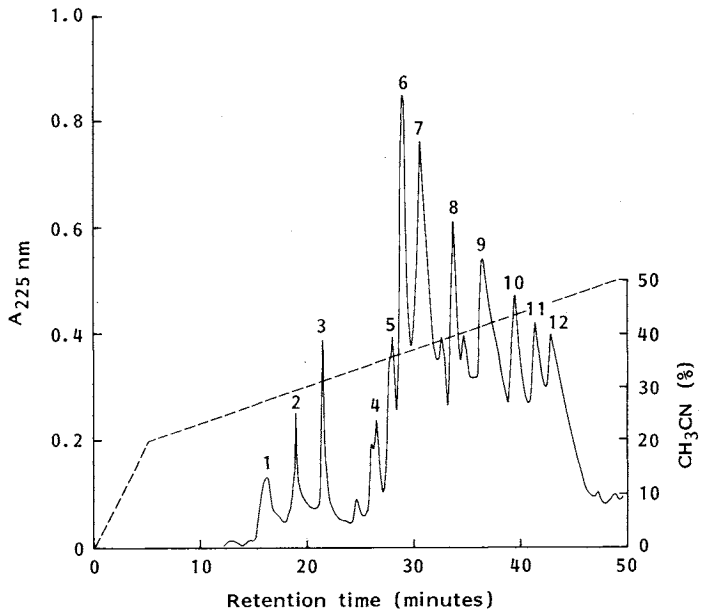
The crude material obtained after chromatography on CM-32 cellulose was further purified by affinity chromatography on immobilized trypsin, as illustrated in Fig. 3. The active fractions eluted with 0.01 N HCl were pooled and lyophilized. The resulting powder was then subjected to preparative HPLC

Fig. 3. Affinity chromatography of crude leupeptin on immobilized trypsin.



After application of the sample, the material on the column (2.2 × 60 cm) was subsequently eluted with 0.4 M NaCl, H_2O and 0.01 N HCl.

Fig. 4. Separation of leupeptin analogues on HPLC as described in Materials and Methods.



chromatography employing a C_{18} (10 μm) column (2.1 × 15 cm) and eluted with a gradient of acetonitrile, as shown in Fig. 4. Of the 12 main peaks, the first 3 were shown to be contaminants, without inhibitor activity. Peaks 4 and 5 were relatively weak inhibitors, and not further characterized. Peaks 6~12 were individually pooled and dried by rotary evaporation at 40°C, under vacuum. Methanol was added and the samples reevaporated in order to remove traces of trifluoroacteic acid and acetonitrile from the samples. Each sample was then dissolved in H_2O and stored for further characterization.

The amino acid contents of peaks 6~12 are listed in Table 1. Most of the components were composed

Table 1. Amino acid compositions of leupeptin analogues.

Peak No.	Amino acids present (mol/mol of peptide)
6	Val (1.9)[a]
7	Thr (2.1)[b]
8	Val, Leu[a]
9	Val (1.8)[a]
10	Leu (2.0)[a]
11	Ile (1.9)[a]
12	Leu (1.8)[a]

[a] The 40 hours hydrolysis time was used.
[b] The 20, 30 and 40 hours hydrolysis times were back-extrapolated to 0 time.

Table 2. Molecular weights of leupeptin analogues.

Peak No.	MW of peptide[a]	MW of alkyl group	Predicted alkyl group
6	463	71	Isobutyryl
7	453	57	Propionyl
9	463	71	*n*-Butyryl
10	477	57	Propionyl
12	491	71	*n*-Butyryl or isobutyryl

[a] The MW's are of the chloride salts.

of hydrophobic single amino acids, *viz.*, Val, Leu or Ile, with the exceptions of peaks 7, which contained Thr as the only amino acid identified, and peak 8, which contained Val and Leu, in equimolar amounts. The material represented by peak 7 is especially important, since a leupeptin analog containing threonine has not been identified to date.

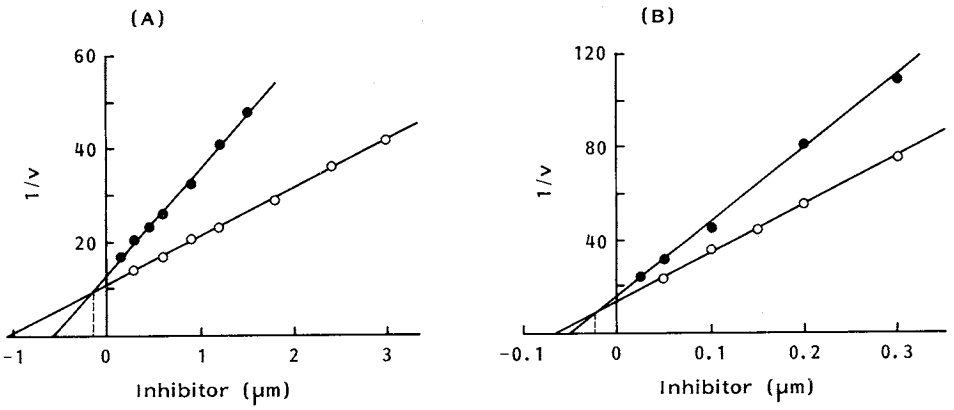
All of the above purified materials showed negative ninhydrin reactions, indicating the presence of blocked amino termini. In addition, all materials were positive in the Sakaguchi reaction and in permanganate oxidation analysis, which in combination with their ability to bind to and inhibit trypsin amidolytic activity, almost certainly shows the presence of argininal at the carboxy-terminus.

The molecular weights of the hydrochloride salts of most of the purified components were determined by mass spectral analysis, and are listed in Table 2. Employing these molecular weights and quantitative amino acid analysis,we found that the amino acids identified in Table 1 were present in a 2:1 molar stoichiometry. With this knowledge, we were able to calculate the molecular weight of the R group present on the amino terminus, and suggest its identity, based on homology with leupeptin. Clearly, the different peaks resolved in Fig. 3, and containing the same amino acid content, represent resolution based on R group differences.

From all of the above information, we conclude that components 6, 7, 9~12 resolved by HPLC in Fig. 3 represent the structures R-X-X-argininal. Peaks 10 and 12, which contain leucine, comigrated with commercial leupeptin in TLC experiments (propanol-H_2O, 7:3)[13]. Peak 8 may be a mixture of two components or may contain a structure of R-X-Y-argininal. This aspect was not investigated further.

Since the leupeptin analogues were purified by affinity chromatography with immobilized trypsin, it would be expected that they would serve as trypsin inhibitors. We found that this inhibition was competitive and reversible and have determined the respective *Ki* values of these inhibitors to trypsin employing Dixon plots, an example of which is provided in Fig. 5. The results obtained for all proteases examined are listed in Table 3. All leupeptin analogues examined were strong inhibitors of trypsin, slightly weaker for plasmin and thrombin, and weakest for activated protein C and factor Xa. Among the four classes of inhibitors, the threonine analogue was the most potent inhibitor against all proteases tested. The inhibitory properties toward trypsin of the Pr-leucine leupeptin analogue were compared with commercial leupeptin (Ac-leupeptin) and the results are illustrated in Fig. 5. The inhibitory potency of the former was approximately 6-fold greater than that of the latter. It is possible that the different acyl groups present contribute to the dissimilarity of their kinetic constants and/or that racemization of the argininal in the commercial preparation occurred during its extraction[1], yielding an amount of Ac-L-Leu-L-Leu-D-argininal,

Fig. 5. Determination of the inhibitor dissociation constants, *Ki*.



(A) Commercial leupeptin with trypsin. (B) Pr-leupeptin isolated in this study. The rate of hydrolysis was calculated from the absorbance increment at 405 nm in 1 minute. The trypsin substrate was S2238 and was used at two different levels, *viz.*, 0.08 mM (●) and 0.16 mM (○).

Table 3. Inhibitor dissociation constants (*Ki*) of leupeptin analogues to different proteases.

Peak No.	*Ki* (μM)				
	Trypsin	Plasmin	Thrombin	APC[a]	Factor Xa
7	0.01	0.20	0.20	0.70	1.31
9	0.02	0.57	0.82	1.78	5.74
10	0.03	0.20	0.55	1.04	2.58
11	0.05	0.75	0.50	1.56	3.53

[a] Refers to activated protein C.

which is inactive.

Discussion

Studies in the literature regarding leupeptin as a proteolytic inhibitor have been conducted mainly with mixtures of Ac-L-Leu-L-Leu-L-argininal and Pr-L-Leu-L-Leu-L-argininal, or with analogues in which Leu is replaced by Ile and Val. In this investigation, we discovered the existence of a new analogue, that composed of threonine instead of leucine and have devised an HPLC method for resolution of leupeptins containing the same amino acid compositions, but different acyl groups on the amino terminus, under mild conditions which do not lead to racemization of the L-argininal. Compared with other leupeptin analogues, that containing threonine displayed a stronger inhibitory activity toward most of the proteases tested. This indicates that a hydrophilic amino acid residue in a leupeptin-like inhibitor might be more suitable for inhibition of serine proteases and provides possibilities for the design of more potent inhibitors.

Leupeptin possesses a broad specificity and is capable of inhibiting serine and thiol proteases, including Ca^{2+}-dependent calpains. The inhibitor dissociation constants (*Ki*) of leupeptin with different serine proteases varies from 7×10^{-9} M with cathepsin B[14] to 8×10^{-5} M with factor IXa[15]. Using α-*N*-tosyl-L-arginine methyl ester and α-*N*-benzoyl-L-arginine ethyl ester as the substrates for trypsin, the *Ki* for leupeptin was found to be 3.4×10^{-7} M and 1.3×10^{-7} M, respectively[13].

Compared with the values from the literature, our *Ki* values for leupeptin and its analogues to trypsin were comparatively low (Table 3). With our mild purification conditions, it is highly unlikely that any racemization of the L-argininal in leupeptin occurred, whereas commercial preparations, prepared by extraction techniques lead to racemization and partial inactivation of leupeptin. This may partly explain

the differences in results. Of the other proteases examined, plasmin and thrombin were more strongly inhibited that factor Xa and activated protein C.

Finally, it has been shown previously that different forms of Ac-leupeptin that arise through hydration and cyclization of the aldehyde group[16], can be resolved by HPLC analysis . When a commercial sample of Ac-leupeptin was analyzed on our system, we observe the same three peaks that have been described for this molecule[16]. Undoubtedly, therefore, some of the shoulders and peaks observed in Fig. 4 represent these isomers. This is the reason that we selected for analysis a limited number of fractions that contained different amino acids. It is likely that under the conditions for mass spectral analysis the isomeric mixture would not be present and a single molecular size would be obtained. In any event, the leupeptins identified would represent a minimal number that likely exist in the crude culture supernate.

In conclusion, we have described a facile method for purifying and resolving different leupeptin analogues of microbial origin. A variety of leupeptin-like inhibitors have been found, differing in amino acid and acyl components. The new analogue, containing threonine, was the most potent inhibitor toward five different serine proteases.

Acknowledgments

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