Purification and Structure of a Novel Cysteine Proteinase Inhibitor, Strepin P-1

Kyoichi OGURA,[†] Mitsuru MAEDA, Masami NAGAI,
Takaharu TANAKA, Kyosuke NOMOTO*
and Takashi MURACHI**

Laboratory of Applied Microbiology, Research Center, Suntory Ltd.
and *Suntory Institute for Bioorganic Research,

Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618, Japan

**Department of Laboratory Medicine, Kyoto University,

Faculty of Medicine,

Kyoto 606, Japan

Received September 5, 1984

Strepin P-1, a new proteinase inhibitor, is a low molecular weight peptide isolated from the culture fluid of *Streptomyces tanabeensis* (SAB-934). Strepin P-1 strongly inhibited not only cysteine proteinases, calpain and papain, but also trypsin. The purification procedures included HP-20 adsorption chromatography, DEAE-cellulose, Amberlite CG-50, Sephadex LH-20 and G-25 column chromatography. The yield was 12 mg from 8 liters of culture fluid. The proteinase inhibitor thus prepared was a peptide composed of tyrosine, valine and argininal, that reacted positively with Sakaguchi and Pauly reagents on TLC. The N-terminal amino acid, tyrosine, was blocked with an isovaleryl group and the structure was elucidated to be *N*-isovaleryl-tyrosyl-valyl-argininal. The amino acid sequence-inhibitory activity relationships of strepin P-1, leupeptin and antipain toward calpain and papain are also discussed.

Proteinous inhibitors acting on proteolytic enzymes have been isolated from the tissues of a variety of animals and plants, and their distribution, properties and biological significance have been studied (see for reviews^{1,2}). These inhibitors are peptides or glycopeptides of high molecular weight over, 5000. Recently, on the other hand, many low molecular weight peptide proteinase inhibitors were found in culture fluids of microbes.^{3~5}) They have been widely used in biological and biochemical studies.^{6,7}) It is also expected that these proteinase inhibitors can be used for diagnostic and therapeutic purposes in some diseases which may be related to aberrations of cellular enzyme activities.^{8,9}) One of the intracellular enzymes which are widely distributed in mammalian and avian cells is calpain [EC 3.4.22.17; Ca^{2+} -dependent cysteine proteinase]; its

biological functions are not fully understood. It may catalyze limited proteolysis of a target protein or proteins in some cells, causing significant changes in metabolic processes.^{11,12}) We attempted to obtain any new proteinase inhibitors of calpain by screening a number of microbes.

This paper describes the isolation and structural elucidation of strepin P-1, a novel proteinase inhibitor from the culture fluid of a strain of a new *Streptomyces* species, *Streptomyces tanabeensis* (SAB-934).

MATERIALS AND METHODS

Strain and culture conditions. Strain SAB-934 isolated from a soil sample collected at the seaside in Tanabe City, Wakayama Prefecture, Japan, was identified as a new species closely related to *Streptomyces galieri* from its culture characteristics and named *Streptomyces*

[†] To whom correspondence should be addressed.

tanabeensis.

The culture filtrate of *Streptomyces tanabeensis* was used for the preparation of a new cysteine proteinase inhibitor, strepin P-1. A seed culture was prepared aerobically at 30°C for 2 days in YM-medium containing 1% glucose, 0.5% polypeptone, 0.3% malt extract and 0.3% yeast extract. A seed culture (300 ml) was inoculated into 8 liters of YM-medium and was grown at 30°C with vigorous aeration in a fermenter (New Brunswick Fermenter). After 48 hr of growth, the culture fluid was harvested by centrifugation.

Assay for strepin P-1. Calpain I and calpain II were purified from porcine erythrocytes and kidney,¹³⁾ respectively. Papain, trypsin and chymotrypsin were purchased from Sigma Chemical Co., ST. Louis, MO, U.S.A. The assay mixture for calpains and papain contained, in a final volume of 2.0 ml, 0.4% casein as a substrate, 50 mM imidazole-HCl buffer (pH 7.2), 5 mM cysteine and 1 mM CaCl₂. The assay mixture for trypsin contained, in a final volume of 1.0 ml, 0.5% casein as a substrate and 50 mM Tris-HCl buffer (pH 7.5). The assay mixture for chymotrypsin contained, in a final volume of 2.0 ml, 0.5% casein as a substrate, 100 mM sodium borate buffer (pH 7.6), 100 mM Na₂CO₃ and 5 mM CaCl₂. For the determination of calpain activity, the protease sample was incubated at 30°C for 30 min and the reaction was stopped by adding 3.0 ml of 5% trichloroacetic acid. The acid soluble products in the filtrate were determined by the method of Ross and Schatz,¹⁴⁾ for which 0.4 ml of the filtrate was diluted with the reagents to a total volume of 2.8 ml, and the absorbance at 750 nm was read against the blank. For the determination of papain, trypsin and chymotrypsin activity, each protease sample was incubated at 35°C for 20 min, and the reaction was stopped by adding 3.0 ml of 5% trichloroacetic acid. The absorbance at 280 nm of the acid soluble products in the filtrate was read against the blank. For the determination of the inhibitory activity of strepin P-1, the inhibitor sample was preincubated in 50 mM imidazole-HCl buffer (pH 7.2) with a fixed amount of calpain, papain, trypsin and chymotrypsin at 30°C for 5 min, and the decrease in proteolytic activity was measured. One unit of strepin P-1 was defined as the amount of the peptide which inhibits 0.015 units of papain as calculated from the straight portion of the inhibition profile, when inhibition of papain was less than 50%.

Oxidation of strepin P-1. The pulverised KMnO₄ (0.5 mg) was added to stirred solution of strepin P-1 (1 mg) in 0.5 ml of *t*-butanol-H₂O (1:1, v/v). After 20 min of reaction, the excess KMnO₄ was decomposed by the addition of methanol. The mixture was filtered and evaporated to dryness under reduced pressure. The residue was dissolved in H₂O, and then the solution was extracted with *n*-butanol.

Digestion with chymotrypsin and carboxypeptidase P.

For the determination of the amino acid sequence, 100 µg each of untreated and oxidized strepin P-1 preparation in 500 µl of buffer was digested with 50 µg each of chymotrypsin (Sigma Chemical Co., St. Louis, MO, U.S.A.) and carboxypeptidase P (Peptide Institute, Protein Research Foundation, Osaka, Japan). The digestion was carried out at 40°C for 90 min in 0.05 M sodium borate buffer (pH 7.6) with chymotrypsin or in 50 mM pyridine-formic acid (pH 2.5) with carboxypeptidase P, and stopped by drying under reduced pressure. The residue was dissolved in 50 µl of 1% NaHCO₃, and 50 µl of 0.25% dansyl chloride (Merck & Co., Rahway, NJ, U.S.A.) was added. After an incubation for 20 min at 40°C, the reaction was terminated by adding 50 µl of glacial acetic acid. The reaction mixture was dried under reduced pressure, and the new N-terminal amino acid was determined on polyamide thin layer chromatography (Cheng Chin Trading Co., Taiwan) by the method of Hartley.¹⁵⁾

Amino acid analysis. Strepin P-1 or oxidized strepin P-1 (approximately 5 µg each) was added to 0.1 ml of 6 N HCl, sealed under reduced pressure, and hydrolyzed at 105°C for 48 hr. Amino acid analysis was performed on a Hitachi model 835 amino acid analyzer.¹⁶⁾

Analytical procedures. The purity of the sample was examined by TLC on a Merck cellulose glass plate (DC Fertigplatten) with *n*-butanol-pyridine-acetic acid-H₂O (15:10:3:12, v/v). Peptides on TLC were stained with ninhydrin, Pauly, Sakaguchi, Schiff and Tollens' reagents. A Gifford model 2000-2 spectrophotometer was used for ultraviolet absorbance measurements. A Hitachi model RMU-6 mass spectrometry system was used for the measurement of the molecular weight of strepin P-1. NMR spectra of strepin P-1 were recorded with a Nicolet NT-360 NMR spectrometer for the analysis of molecular formula.

Chemicals. Leupeptin (acetyl-leucyl-leucyl-argininal) and antipain (*N*-[(*S*)-1-carboxy-2-phenylethyl]carbamoyl-arginyl-valyl-argininal) were purchased from the Peptide Institute, Protein Research Foundation, Osaka, Japan. Mitsubishi Diaion HP-20 was purchased from Mitsubishi Chemical Industries Ltd., Tokyo, Japan. DEAE-cellulose (DE-23) was purchased from Whatman Ltd., Springfield, U.K. Amberlite CG-50 was the product of Rhom and Hass Ltd., U.S.A. Sephadex LH-20 and G-25 were the products of Pharmacia, Upsala, Sweden. Other reagent-grade chemicals were obtained from Wako Pure Chemicals Ltd., Osaka or Nakarai Chemicals Ltd., Kyoto, Japan.

RESULTS

Purification of strepin P-1

When Streptomyces tanabeensis (SAB-934)

was cultured in YM-medium, the activity of strepin P-1 in the culture fluid increased rapidly during the exponential phase of cell growth and reached a maximum in the stationary phase after 40 hr of incubation at 30°C, followed by a rapid decline under the culture conditions used.

After removing the cells by centrifugation from the culture fluid in the stationary phase, the supernatant (8 liters) was applied to a column of HP-20 (16 × 17 cm). The column was washed with 25 liters of water and then with 10 liters of methanol. The latter eluate, which contained strepin P-1 activity, was concentrated to 200 ml, and the concentrate was

put on a column of DE-23 (OH⁻ form, 6 × 10 cm). The inhibitory activity appeared in the flow-through fraction and the subsequent washings with water. By this chromatography, most of the brown color of the culture fluid could be removed from the inhibitor fraction. The effluent and washings were combined and immediately applied to a column of Amberlite CG-50 (H⁺ form, 6 × 5 cm). The column was washed with water, followed by 0.2 M acetic acid, until the absorbance at 250 nm decreased to nearly zero. The inhibitor was then eluted with 500 ml of 1 M pyridine-acetic acid buffer (pH 5.0) and lyophilized. The lyophilized sample was dissolved in 5 ml of 50%

FIG. 1. Chromatography of Strepin P-1 Crude Preparation on Sephadex LH-20 (A), and Sephadex G-25 (B).

For A, a column of Sephadex LH-20 (2.8 × 185 cm) was loaded with 263 mg in 5 ml and developed with 50% MeOH-acetic acid (100:3, v/v) at a flow rate of 50 ml/hr. Fractions of 10 ml each were collected. (○—○), absorbance at 280 nm; (●—●), papain-inhibitory activity. For B, the active fractions from Sephadex LH-20 were combined and evaporated under reduced pressure. The evaporated preparation (53 mg in 3 ml of H₂O) was put on a column of Sephadex G-25 (2 × 45 cm). The column was washed with H₂O at a flow rate of 40 ml/hr until the absorbance at 280 nm decreased to nearly zero. At the point indicated by an arrow in the figure, H₂O was supplemented with 0.2 M acetic acid. Inhibitory activity was eluted as a single peak. Fractions of 6 ml each were collected. (○—○), absorbance at 280 nm; (●—●), papain-inhibitory activity.

TABLE I. PURIFICATION OF STREPIN P-1 FROM CULTURE FLUID

Step	Dry weight (mg)	Total activity ($\times 10^{-4}$ units)	Yield (%)	Specific activity (units/mg)	Purification (-fold) ^c
Culture fluid ^a	n.d. ^b	384	100		
HP-20	13,400	280	73	209	1.00
DEAE-cellulose	5,710	160	42	280	1.34
Amberlite CG-50	263	80	21	3,040	14.5
Sephadex LH-20	53	62	16	11,700	56
Sephadex G-25	12.1	49	13	40,500	194

^a From 8 liters of culture fluid.^b Total dry weight of this fraction could not be determined.^c A specific activity of 209 units/mg found at the 2nd step was taken as unity.

methanol-acetic acid (100:3, v/v) and insoluble material was removed by centrifugation. The supernatant was put on a Sephadex LH-20 column (2.8 $\times$ 185 cm), and the column was washed with the same solvent mixture as above at a flow rate of 50 ml/hr under monitoring at 280 nm (Fig. 1A). Fractions which showed papain-inhibitory activity were combined and dried under reduced pressure. The residue was dissolved in 3 ml of water and was put on a column of Sephadex G-25 (2 $\times$ 45 cm) swelled in water. The column was washed with water until the absorbance at 280 nm decreased to nearly zero. It was then washed with 0.2 M acetic acid, at a flow rate of 40 ml/hr, which eluted the papain-inhibitory activity as a sharp and symmetrical peak (Fig. 1B). The purification procedures are summarized in Table I. Examination of the purity of strepin P-1 by TLC revealed that the residue from the active fractions (No. 125~127 in Fig. 1B) showed only one spot positive to Pauly and Sakaguchi reagents. Paper electrophoresis at pH 6.4 also indicated the homogeneity of this preparation; it moved toward the cathode with a mobility almost equal to that of arginine.

Properties and structure of strepin P-1

The absorption spectrum of strepin P-1 in neutral pH solution had a maximum at 278 nm and a trough at 245 nm. The infrared spectrum in KBr disc exhibited absorption bands at 3300~3200 cm^{-1} (NH and OH), 2390 cm^{-1} (CH_2 and CH), 1630 cm^{-1} (amide and gua-

TABLE II. AMINO ACID COMPOSITION OF NATURAL AND OXIDIZED STREPIN P-1^a

Amino acid	Natural strepin P-1	Oxidized strepin P-1 with KMnO_4
Valine	1.00 (1)	1.00 (1)
Tyrosine	0.996 (1)	0.946 (1)
Arginine	0 (0)	0.873 (1)

^a All values were calculated assuming one valine residue present per molecule of strepin P-1.

nidium), 1540 cm^{-1} (amide II) and 1500 cm^{-1} (phenyl). Strepin P-1 also gave positive color reaction on TLC to Pauly, Sakaguchi and Tollens' reagents, but negative to ninhydrin reagent. The amino acid analysis of untreated strepin P-1 showed equal amounts of valine and tyrosine, while after KMnO_4 -oxidation one molecule of arginine appeared (Table II).

The molecular formula of strepin P-1 was assigned as $\text{C}_{25}\text{H}_{40}\text{O}_5\text{N}_6$ (M.W. 504) on the basis of elemental analysis. *Anal.* Calcd. for $\text{C}_{25}\text{H}_{40}\text{O}_5\text{N}_6$: 4 H_2O ; C, 52.07; H, 8.73; N, 14.75; Found, C, 52.37; H, 8.51; N, 14.82 and the field desorption mass spectrum (FD-MS) [m/z 505 ($\text{M}+1$)⁺]. The melting point and specific rotatory power of strepin P-1 were: mp 184~186°C (decomposition) and $[\alpha]_D^{24} -10.9^\circ$ ($c=0.14$, H_2O).

From these results, we concluded that strepin P-1 is a tripeptide containing tyrosine, valine and argininal, and that the N-terminal amino group in the peptide must be blocked.

TABLE III. ^1H -NMR SPECTROSCOPIC DATA FOR STREPIN P-1

Residue	Signal (δ ppm)*					
Isovaleryl- $\begin{array}{c} \text{CH}_3 \\ \\ \text{CH}_3-\text{CH}-\text{CH}_2-\text{CO}- \\ \\ \text{CH}_3 \end{array}$	0.80 3H d $J=6.5$	0.83 3H d $J=6.5$	1.50 1H m	2.09 2H m		
Valyl- $\begin{array}{c} \text{CO} \\ \\ \text{CH}_3-\text{CH}-\text{CH} \\ \quad \\ \text{CH}_3 \quad \text{NH} \end{array}$	0.90 3H d $J=6.5$	0.94 3H d $J=6.5$	1.70~1.90 1H m	4.02 1H d $J=8$		
Tyrosyl- $\begin{array}{c} \text{CO} \\ \\ \text{HO}-\text{C}_6\text{H}_4-\text{CH}_2-\text{CH} \\ \\ \text{NH} \end{array}$	2.90 1H dd $J=14, 9.2$	3.28 1H dd $J=14, 3.5$	4.48 1H dd $J=9.2, 3.5$	6.82 2H d $J=9$	7.13 2H d $J=9$	
Argininal $\begin{array}{c} \text{NH}_2 \\ \\ \text{NH}=\text{C}-\text{NH}-(\text{CH}_2)_3-\text{CH} \\ \quad \\ \text{NH} \quad \text{CHO} \end{array}$	1.70~1.90 4H m	2.95~3.25 2H m	3.50~3.95 1H m	4.96 0.5H d $J=4$	5.42 0.5H d $J=3$	

* Chemical shifts were measured from internal DSS.

The ^1H -NMR spectrum of strepin P-1 in D_2O was analyzed by comparison with that of the known proteinase inhibitor, leupeptin. The ^1H -NMR spectrum indicated the presence of equimolar amounts of isovaleryl, tyrosine, valine and argininal moieties, and provided further evidence for the existence of hydrate and carbinolamine forms in the argininal moiety (Table III); this had been shown to hold for leupeptin.¹⁷⁾

The amino acid sequence of strepin P-1 was determined using oxidized strepin P-1 as the starting material. After digestion with chymotrypsin and dansylation, the appearance of dansyl-valine was confirmed on polyamide TLC. Furthermore, the result of carboxypeptidic cleavage suggested that the carboxyl-terminal sequence of the oxidized strepin P-1 should be valyl-arginine; hence, tyrosyl-valyl-argininal for the natural strepin P-1 (Fig. 2).

Reactivity

The purified strepin P-1 inhibited calpain I, calpain II and papain strongly (Fig. 3). The doses of strepin P-1 required for 50% inhibition, ID_{50} , toward calpain I from porcine

FIG. 2. Chemical Structure of Strepin P-1.

erythrocytes, calpain II from porcine kidney and papain under the conditions employed were $0.38 \mu\text{g}/\text{tube}$, $0.56 \mu\text{g}/\text{tube}$ and $0.20 \mu\text{g}/\text{tube}$, which corresponded to $7.5 \times 10^{-7} \text{M}$, $1.1 \times 10^{-6} \text{M}$ and $2.0 \times 10^{-7} \text{M}$, respectively. As shown in Fig. 3, ID_{50} values of leupeptin were $7.0 \times 10^{-7} \text{M}$ for calpain I, $9.4 \times 10^{-7} \text{M}$ for calpain II and $2.1 \times 10^{-6} \text{M}$ for papain. ID_{50} values of antipain were $4.3 \times 10^{-6} \text{M}$ for calpain I, $9.9 \times 10^{-7} \text{M}$ for calpain II and $6.0 \times 10^{-7} \text{M}$ for papain. Strepin P-1 also inhibited trypsin with an ID_{50} value of $2.0 \times 10^{-6} \text{M}$ (graphic data not shown).

DISCUSSION

The primary object of this study was to

FIG. 3. Effects of Strepin P-1, Leupeptin and Antipain on Calpain I (A), Calpain II (B) and Papain (C).

Each tube contained 0.33 unit of calpains I and II, or 0.015 unit of papain. After preincubation with various amounts of each inhibitor at 30°C or 35°C for 5 min, the remaining activity was assayed. (●—●), streptin P-1; (○—○), leupeptin; (△—△), antipain.

isolate some novel inhibitor acting on calpain from the culture fluid of certain microbes. We were able to isolate streptin P-1, which has a structure unprecedented among the known proteinase inhibitors from microbes. However, the specificity spectra of streptin P-1 toward several cysteine and non-cysteine proteinases were not so unusual as we had expected: it did inhibit calpain strongly, but also papain fairly strongly and even trypsin.

The presence of a C-terminal argininal residue is, of course, responsible for its inhibition of trypsin. Both leupeptin and antipain also contained argininal residues at the carboxyl end,^{17,18)} which may be recognized as P1 residue by the enzyme, if one uses the subsite notation originated by Schechter and Berger.¹⁹⁾ Trypsin may not be influenced pro-

foundly by the nature of P2 residue, whereas the latter residue plays an important role in determining specificity of cysteine proteinases. Papain favors a valine at P2 position over a leucine residue, while the reverse is true with calpains, both I and II.²⁰⁾ The ID_{50} values found in the present study (Fig. 3) are in full accord with these known orders of preference. Thus, streptin P-1 as well as antipain, which have a valine residue at P2 position, inhibited papain more strongly than calpain, while leupeptin with a leucine at P2 position inhibited calpain more strongly than papain.

The presence of a tyrosine residue at P3 position was totally unexpected, because none of the previously known proteinase inhibitor peptides contained tyrosine.^{17,18,21)} Because of these novelties, we conducted rather thorough structural studies on streptin P-1, and all the data (Fig. 2, Tables II and III) confirmed the unique structure of this new inhibitor as *N*-isovaleryl-tyrosyl-valyl-argininal. A synthetic peptide based on this proposed amino acid sequence, is fully active as the cysteine proteinase inhibitor. Further studies on the mechanism of inhibition and biosynthesis of streptin P-1 are to be conducted in comparison with those of leupeptin and antipain.³⁾

Acknowledgment. We thank Dr. Ichiro Kubota for performing the amino acid analysis and Dr. Naoki Higuchi for his valuable comments.

REFERENCES

- 1) R. Vogel, I. Trautschold and E. Werle, "Natural Proteinase Inhibitor," Academic Press, New York, N.Y., 1968.
- 2) M. Laskowski, Jr. and I. Kato, *Ann. Rev. Biochem.*, **49**, 593 (1980).
- 3) H. Umezawa, "Methods in Enzymology," Vol. 45, ed. by L. Lorand, Academic Press, New York, N.Y., 1976, p. 678.
- 4) T. Aoyagi, "Bioactive Peptides Produced by Microorganisms," ed. by H. Umezawa, T. Shibata and T. Takita, Kodansha Scientific Press, Tokyo, 1978, p. 129.
- 5) S. Murao, *Nippon Nôgeikagaku Kaishi*, **55**, 503 (1981).
- 6) C. Devaux, P. Corvol and J. Menard, *Biochim. Biophys. Acta*, **359**, 421 (1974).

- 7) K. Murakami and T. Inagami, *Biochem. Biophys. Res. Commun.*, **62**, 757 (1975).
 - 8) P. Libby and A. L. Goldberg, *Science*, **199**, 534 (1978).
 - 9) R. P. Miller, C. J. Poper, C. W. Wilson and E. Devoit, *Biochem. Pharmacol.*, **21**, 2941 (1972).
 - 10) T. Aoyagi, M. Ishizuka, T. Takeuchi and H. Umezawa, *Japan. J. Antibiotics Suppl.*, **30**, s-121 (1977).
 - 11) T. Murachi, "Calcium and Cell Function," Vol. 4, ed. by W. Y. Chung, Academic Press, New York, N.Y., 1983, p. 377.
 - 12) T. Murachi, *Trends in Biochem. Sci.*, **8**, 167 (1983).
 - 13) A. Kitahara, T. Sakai, T. Kikuchi, N. Yumoto, N. Yoshimura, M. Tanaka and T. Murachi, *J. Biochem.*, **95**, 1767 (1984).
 - 14) E. Ross and G. Schatz, *Anal. Biochem.*, **54**, 304 (1973).
 - 15) B. S. Hartley, *Biochem. J.*, **119**, 805 (1970).
 - 16) H. Speckman, H. W. Stein and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
 - 17) T. Aoyagi, T. Takeuchi, A. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada, K. Maeda and H. Umezawa, *Japan. J. Antibiotics*, **22**, 283 (1969).
 - 18) H. Suda, T. Aoyagi, M. Hamada, T. Takeuchi and H. Umezawa, *Japan. J. Antibiotics*, **25**, 263 (1972).
 - 19) I. Schechter and A. Berger, *Biochem. Biophys. Res. Commun.*, **27**, 157 (1967).
 - 20) T. Sasaki, T. Kikuchi, N. Yumoto, N. Yoshimura and T. Murachi, *J. Biol. Chem.*, **259** in press.
 - 21) T. Shin-Watanabe, K. Fukuhara and S. Murao, *Tetrahedron*, **38**, 1775 (1982).
-