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## A Specific Protease Inhibitor from *Streptomyces*

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A protease inhibitor was purified 700-fold from culture broth of *Streptomyces* by butanol extraction, washing with ether, and chromatographies on silica gel, SP-Sephadex, and Bio-Gel P-2. This material specifically inhibited trypsin as well as a tissue-type plasminogen activator, but it only slightly inhibited the other type of plasminogen activator, urokinase, and did not inhibit other proteases tested.

**Keywords**—inhibitor; *Streptomyces*; plasminogen activator; trypsin; low-molecular-weight inhibitor

Various protease inhibitors have been isolated from animal tissues, blood plasma, plants and microorganisms. They differ from each other in molecular weight and specificity. Inhibitors of microbial origin have attracted particular attention.<sup>1)</sup> They are mostly small molecules, and some have high and some have rather low specificity. These inhibitors may be useful not only in providing clues to the mechanisms of various enzymic reactions, but also in the treatment of diseases.

Recently it has been reported that plasminogen activator(s) enhances cell transformation<sup>2)</sup> and is involved in the metastasis of cancer. Antibodies to plasminogen activator suppressed the metastasis of a human tumor.<sup>3)</sup> We therefore attempted to obtain low-molecular-weight inhibitors of plasminogen activator from a microorganism.

An inhibitor of plasminogen activator was purified from culture broth of *Streptomyces*. The activity was determined with a tissue-type plasminogen activator (t-PA) from pig heart. The inhibitory activity in broth increased with culture time and reached the maximum value after the fourth day of culture. The inhibitor was purified according to the procedure described under Experimental. As shown in Table I, we achieved 700-fold purification with a

TABLE I. Purification of the Inhibitor

	Dry weight (mg)	IC <sub>50</sub> <sup>a)</sup> (mg/ml)	Purification (-fold)	Yield (%)
Broth	462000	35	1	
Butanol extraction	14000	1.1	32	96
Washing with ether	8400	0.8	44	80
SiO <sub>2</sub> -chromatography	2079	0.3	116	52
SP-Sephadex chromatography	614	0.15	233	31
Gel-filtration	185	0.05	700	28

a) The concentration required to inhibit t-PA (0.5 IU/ml) by 50%.

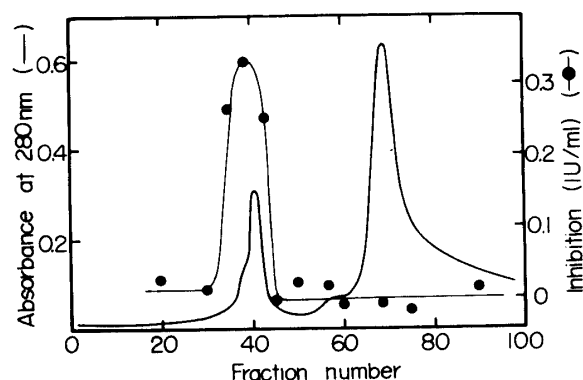


Fig. 1. A Chromatogram on Bio-Gel P-2 of the Active Fraction Obtained from SP-Sephadex Chromatography

A 500  $\mu$ l aliquot of 20 mg/ml active fraction was applied to a column of Bio-Gel P-2 (1.0  $\times$  28 cm). Elution was performed at 4  $^{\circ}$ C with distilled water, and fractions of 500  $\mu$ l were collected. The UV absorption was measured at 280 nm (—). The inhibition of t-PA by each fraction (—●—) was determined as follows. A 50  $\mu$ l aliquot was added to 25  $\mu$ l of t-PA solution (5.0 IU/ml), and then a solution of 175  $\mu$ l of 0.14 mg/ml fibrinogen, 3.6 CU/ml plasminogen and 0.29 NIH units/ml thrombin in 50 mmol/l Tris-HCl buffer containing 150 mmol/l NaCl, pH 7.4, was further added. The mixture was incubated at 25  $^{\circ}$ C for 2 h. The inhibition was expressed in terms of the decrease of activity of the plasminogen activator, which was determined by the method described under Experimental.

yield of 28%. As the inhibitor was more soluble in butanol than in water and was not soluble in ether, it may be assumed that this material is moderately hydrophobic. The profile of the final step of the purification on Bio-Gel P-2 is shown in Fig. 1. The inhibitory activity was exclusively observed in fractions 35–45, and was not associated with the retarded main fraction. Since the inhibitory activity was not detected in the void fraction (the fraction number 14–15), the inhibitor should have a small molecular weight in the range of several hundred at most. The purity of the inhibitor was investigated by thin layer chromatography, using two different solvent systems, chloroform–methanol = 3 : 1 and 1 : 1. In both cases, only one spot was visualized with phosphorus-molybdic acid, which develops all organic compounds. The result indicates high purity of the inhibitor preparation. The purified material gave a positive Rydon–Smith reaction,<sup>4)</sup> but a negative ninhydrin reaction. It was soluble in water, methanol, and butanol, but not soluble in acetone, chloroform, or ether. This material did not have specific ultraviolet (UV) absorption, and the absorption was increased monotonically with decreasing wavelength to 220 nm. The infrared (IR) spectrum showed that this material did not contain peptide bonds. Further experiments to obtain information about the structure of the inhibitor are in progress.

There are two different types of plasminogen activators, *i.e.*, tissue-type and urokinase (UK)-type plasminogen activators. They differ from each other in a number of respects including molecular weight, serological reactivity, and requirement of fibrin for activation.<sup>5)</sup> It was of interest to know whether this inhibitor could specifically inhibit one type of plasminogen activator. As shown in Fig. 2, this inhibitor rather specifically inhibited t-PA (fluorometric assay method). A similar result was obtained using the fibrin plate method, in which the inhibition of UK was almost negligible. When a t-PA from Ehrlich ascites carcinoma was used in place of that from pig heart, the inhibition was still clear. There are several different kinds of inhibitors of plasminogen activators in blood plasma,<sup>6)</sup> but an inhibitor which acts specifically on one type of plasminogen activator has not previously been reported. Kruithof *et al.*<sup>6)</sup> have suggested that an antiactivator with apparent molecular weight of 40000 forms complexes with both types of plasminogen activator and inhibits them. Loskutoff's group has isolated antiactivators with a molecular weight of 50000 from cultured bovine aortic endothelial cells<sup>7)</sup> and from human platelets,<sup>8)</sup> but these antiactivators also inhibited both types of plasminogen activator. On the other hand, it has been reported that proteins isolated from human monocytes<sup>9)</sup> and from seeds of a legume<sup>10)</sup> specifically inhibit UK and t-PA, respectively.

Further experiments were conducted to investigate the specificity of this inhibitor toward various types of proteases. The concentrations of proteases used were set either to give the

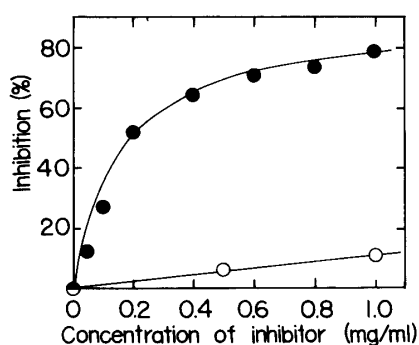


Fig. 2. The Inhibition of Two Different Plasminogen Activators by the Inhibitor

Various concentrations of the inhibitor (obtained from the SP-Sephadex chromatography step) were added to 0.5 IU/ml (final concentration) t-PA or UK. The inhibition of t-PA or UK was determined by the method described in Fig. 1 (—●— and —○—, respectively).

TABLE II. Effect of the Inhibitor on Various Proteases

Enzyme	Inhibition
Tissue-plasminogen activator	80%
Urokinase	12%
Trypsin	100%
Thrombin	No inhibition
Plasmin	No inhibition
$\alpha$ -Chymotrypsin	No inhibition
Subtilisin	No inhibition
Pepsin	No inhibition
Papain	No inhibition
Carboxypeptidase B	No inhibition
Plasminogen activator in Ehrlich ascites carcinoma	75%

The inhibition was expressed in terms of the percentage decrease of the activity, which was determined by the methods described under Experimental. The concentration of the inhibitor was fixed at 1 mg/ml. The concentrations of trypsin, plasmin and thrombin were set to give about the same amidolytic activity (39 pmol of 7-amino-4-methylcoumarine/min). The concentrations of  $\alpha$ -chymotrypsin, pepsin, papain, subtilisin and carboxypeptidase B were set at 0.05 mg/ml.

same activity or the same concentration, and the concentration of the inhibitor was fixed at 1 mg/ml in all experiments. As is shown clearly in Table II, this inhibitor acted not only on t-PA, but also on trypsin. Trypsin was in fact more susceptible to this inhibitor than t-PA. Kinetic experiments will be necessary to elucidate the mechanism of this inhibition. Other serine proteases, including plasmin, thrombin, and chymotrypsin were not inhibited at all. Neither was pepsin (an acid protease), papain (an SH-protease), or carboxypeptidase B (a metalloprotease).

The above findings reveal a very peculiar inhibitory spectrum. The only analogy so far found is an inhibitor isolated from seeds of a legume.<sup>10)</sup> This inhibitor, however, is a protein with a molecular weight of 20000. Furthermore, this is essentially a trypsin inhibitor; the concentration of the inhibitor required to inhibit t-PA by 50% was  $1.1 \times 10^{-7}$  mol/l, while that in the case of trypsin was only  $6.9 \times 10^{-10}$  mol/l.

In the present paper we have described the isolation of a low-molecular-weight inhibitor of t-PA and trypsin from culture broth of *Stereptomyces*.

### Experimental

Human fibrinogen (93% clottable), thrombin (12.5 NIH units/mg), plasminogen (3.5 CU/mg) and urokinase (450 IU/mg) were obtained from Green Cross Co. Trypsin, papain and carboxypeptidase B were purchased from Sigma Chemical Co. Subtilisin was obtained from Nagase Biochemicals Ltd. Synthetic substrates, *tert*-butyloxycarbonyl (Boc)-Val-Leu-Lys-4-methylcoumaryl-7-amide (MCA) and Boc-Val-Pro-Arg-MCA were obtained from the Peptide Institute Inc. A tissue-type plasminogen activator was partially purified from pig heart.<sup>11)</sup> Ehrlich ascites carcinoma was solubilized with 0.5% Triton X-100 to determine the fibrinolytic activity. Chymotrypsin was kindly provided by Dr. Matsushima (Tokyo Institute of Technology).

**Determination of Enzymic Activity**—The activity of plasminogen activator was determined by measuring the quantity of plasmin formed from plasminogen, using Boc-Val-Leu-Lys-MCA as the substrate.<sup>12)</sup> The activities of trypsin and thrombin were determined, with Boc-Val-Leu-Lys-MCA and Boc-Val-Pro-Arg-MCA, respectively, as substrates under the same conditions as used for the determination of plasmin. The activities of chymotrypsin and subtilisin were measured spectrophotometrically with *N*-acetyl-L-tyrosine ethyl ester as the substrate.<sup>13)</sup> Pepsin and carboxypeptidase B were determined with acetyl-L-Phe-L-diiodotyrosine<sup>14)</sup> and hippuryl-L-arginine,<sup>15)</sup> respectively,

as substrates. The activity of papain was measured according to a reported method.<sup>16)</sup> Fibrinolytic activity of Ehrlich ascites carcinoma was measured using fibrin plates prepared for fibrin autography.<sup>17)</sup>

**Purification of Protease Inhibitor**—*Streptomyces*, strain MH194-hF2, was cultured at 27°C in a stainless steel fermenter of 15 l volume in a medium containing 1.0% soypepton, 2.0% galactose, 0.5% corn steep liquor, 2.0% dextrin, 0.2% ammonium sulfate, and 0.2% CaCO<sub>3</sub>. The culture filtrate (13.4 l) was extracted with an equal volume of butanol. The butanol extract was evaporated under a vacuum, and the crude powder was washed with a mixture of ethyl ether and petroleum ether (1:2). As the inhibitor did not dissolve in the mixture, soluble impurities were removed. The inhibitor was further purified by chromatography on a silica gel column. The sample was dissolved in 20 ml of methanol, and 20 g of silica gel was added. The mixture was evaporated under a vacuum, and then applied to a silica gel column (100 g) equilibrated with chloroform. The column was washed with chloroform-methanol (50:1), and then eluted with chloroform-methanol (5:1). The active fractions were combined and evaporated under a vacuum. The inhibitor was further purified by SP-Sephadex chromatography. The sample was dissolved in distilled water immediately prior to application to an SP-Sephadex column (300 ml) equilibrated with distilled water, and the bound materials were eluted with a 0–1 mol/l NaCl gradient. The active fraction began to be eluted with 0.2 mol/l NaCl. The active fractions were pooled and evaporated under a vacuum. It was subsequently purified by chromatography on a Bio-Gel P-2 column (1.0 × 28 cm) equilibrated with distilled water.

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