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PURIFICATION AND PROPERTIES OF A PROTEINACEOUS METALLO-PROTEINASE INHIBITOR FROM *STREPTOMYCES NIGRESCENS* TK-23

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Summary

A novel metallo-proteinase inhibitor which is capable of inhibiting the activities of metallo-proteinases such as thermolysin, was isolated from the culture filtrates of *Streptomyces nigrescens* TK-23. The inhibitor was purified batch-wise from the culture filtrate by Amberlite IRC-50 and column chromatographies on CM-Sephadex C-50 and Sephadex G-50. The purified inhibitor showed a single band on 15% polyacrylamide gel electrophoresis at pH 4.3, and at pH 7.5 on SDS-gels. The inhibitor retained 80% of its original activity after treatment of 100°C for 5 min between pH 2 and 7. The molecular weight was estimated to be 12 000 by gel filtration and SDS-polyacrylamide gel electrophoresis, and calculated as 11 950 from its amino acid composition. The isoelectric point was pH 10.3. The inhibitor showed a high content of hydrophobic amino acids, did not contain tryptophan, and had two disulfide bridges. It also showed specific inhibitory activity for metallo-proteinases but not for serine-, thiol- and carboxyl-proteinases.

Introduction

Kunitz and Northrop [1] isolated and crystallized the trypsin-kallikrein inhibitor in 1936. Since then, a number of proteinaceous serine proteinase inhibitors [2—5] have been isolated and studied in a variety of plants and animals. Amino acid sequences have been determined for many of these inhibitors. Furthermore, three-dimensional structures were established for the basic pancreatic trypsin inhibitor (Kunitz [6]), soybean trypsin inhibitor [7], and *Streptomyces* subtilisin inhibitor [8].

These studies of proteinaceous serine proteinases inhibitors have been carried out to investigate their mechanism, physiological role, and pharmacological significance. However, little is known about the metallo-proteinase inhibitors, despite their wide occurrence, e.g, snake toxin proteinase, pancreatic carboxypeptidase A and leucine aminopeptidase, thermolysin (obtained from *Bacillus thermoproteolyticus*), *Pseudomonas aeruginosa* proteinase [9], and *Clostridium histolyticum* collagenase [10].

During investigations of proteinase inhibitors [11—13] in microorganisms, we [14] isolated a potent proteinaceous metallo-proteinase inhibitor from the culture filtrate of *S. nigrescens* (strain TK-23).

In this communication, we describe the isolation, characterization and inhibitory spectrum of this inhibitor.

Materials and Methods

Organism. Streptomyces nigrescens TK-23, isolated from a soil sample, was used throughout these experiments. This strain was grown and maintained on a agar medium containing 0.4% yeast extract, 1% malt extract and 0.4% glucose (pH 7.3).

Enzymes and substrates. Thermolysin (3 times crystallized) was kindly supplied by Daiwa Kasei (Japan). The crystalline metallo-proteinases of *P. aeruginosa* IFO 3455 [9], *B. subtilis* var. *amylosacchariticus* [15], *B. amyloliquefaciens* [16] and *Serratia sp.* E-15 [17] were kindly donated by Dr. K. Morihara (Shionogi Co.), Dr. D. Tsuru (Nagasaki University) and Dr. K. Tomoda (Takeda Chemical Industries), respectively. The metallo-proteinase of *B. subtilis* YT-25 [18] was purified in this laboratory. Bovine pancreas carboxypeptidase A (twice crystallized), pig leucine aminopeptidase (chromatographically purified), and *C. histolyticum* collagenase (ammonium sulfate fraction) were purchased from Sigma Chemical Co.

Human pancreatic elastase (acetone powder of human pancreatic juice) was kindly supplied by Toyo Jozo Co. Bovine pancreatic trypsin (twice crystallized), bovine pancreatic α-chymotrypsin (3 times crystallized), and hog pancreas elastase (twice crystallized) were purchased from Sigma Chemical Co. Crystalline subtilisin BPN′ was purchased from Nagase Sangyo Co. (Japan).

Papain (twice crystallized) and Ficin were purchased from Sigma Chemical Co. and Wako Pure Chemical Co., respectively.

Carboxyl proteinases of *Rhodotorula glutinis* [19], *Cladosporium sp.* [20] and *Scytalidium lignicolum* (A and B) [21] were purified in this laboratory.

The substrates, benzyloxycarbonyl-L-glycyl-L-leucine amide (Z-Gly-Leu · NH_2), *N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt), and hippuryl-L-phenylalanine were purchased from the Peptide Research Foundation (Osaka, Japan). *N-t*-Butoxycarbonyl-L-alanine-*p*-nitrophenyl ester (Boc-Ala-ONp) was from Sigma Chemical Co., Azocoll (50—100 mesh) from Calbiochem (USA) and casein from Wako Pure Chemical Co. (Japan).

Culture conditions. S. nigrescens TK-23 was cultured in 500-ml flask containing 100 ml medium (pH 7) consisting of 1% glucose, 1% meat extract, 3% polypeptone, 0.1% K_2HPO_4, 0.1% NaCl. The flasks were shaken at 30°C. The inhibitor began to accumulate at approx. 20 h after the beginning of cultiva-

tion and its amount in the culture fluid increased almost linearly with time to reach a maximum at 50 h. The culture fluid at 48-h cultivation was used for the preparation of the inhibitor.

Assay of proteinase and inhibitor activities

(1) Assay of metallo-proteinase activity. (a) with casein substrate: A mixture of the enzyme and inhibitor in 1 ml 50 mM Tris-HCl buffer (pH 7.5) was incubated at 37°C for 10 min, and then 3 ml 1.33% casein was added. After incubation at 37°C for 10 min, 4 ml 0.44 M trichloroacetic acid was added. The resulting suspension was allowed to stand at 37°C for 20 min. To 1 ml filtrate, 5 ml 0.44 M Na_2CO_3 and 1 ml Folin reagent were added, the mixture was kept at 37°C for 20 min, and the absorbance measured at 660 nm. When the difference between absorbance with and without inhibitor at 660 nm was less than 0.5, its value was proportional to the concentration of inhibitor. One inhibitor unit was defined as the amount of inhibitor that caused a 50% reduction of its caseinolytic activity in this assay system. (b) With Z-Gly-Leu · NH_2 as substrate [22]: The reaction mixture consisted of 2 μmol Z-Gly-Leu · NH_2, 1 μg enzyme, and various amounts of inhibitor, in 1 ml 10 mM Tris-HCl buffer (pH 7.0), 10 mM $CaCl_2$. After 20-min incubation at 37°C, the mixture was cooled in an ice-bath and 0.5 ml 3.72 M sodium acetate buffer (pH 5.3), 0.2 mM NaCN, 0.5 ml ninhydrin (3%) in methylcellosolve, was added. The mixture was placed in a boiling water-bath for 15 min; immediately after that 5 ml 50% (v/v) isopropyl alcohol were added and the absorbance at 570 nm was read, after having cooled the mixture to room temperature. (c) Carboxypeptidase A was measured spectrophotometrically in 25 mM Tris-HCl buffer (pH 7.5), 0.5 M NaCl, at 25°C, using 1 mM hippuryl-L-phenylalanine [23]. (d) Leucine aminopeptidase was assayed at pH 7.5 and 25°C with 50 mM L-leucine amide as substrate [24]. (e) Collagenase activity was assayed at pH 7.5 and 37°C with 4 mg/ml Azocoll as substrate [25].

(2) Assay of serine, thiol and carboxyl proteinases. (a) Trypsin, chymotrypsin, subtilisin, papain, ficin, and carboxyl proteinases (pepsin, etc.): inhibitory action against these proteinases was estimated by the same assay system using casein as substrate. Buffer and enzyme concentrations were adjusted to fit the requirements. Papain was used after activation with cystein and EDTA. (b) Kallikrein: esterase activity of kallikrein was spectrophotometrically assayed at pH 7.5 and 25°C, with 1 mM benzoyl-L-arginine ethyl ester as substate [26]. (c) Elastase: esterase activity of elastase was determined photometrically by the method of Visser and Blout [27] with 20 mM *N-t*-butoxylcarbonyl-L-alanine-*p*-nitrophenyl ester as substrate.

Determination of protein concentration

Protein content was assayed spectrophotometrically assuming that $E^{1\%}_{1cm}$ at 280 nm was 5.56, which was obtained from the dry weight of the purified inhibitor preparation.

Isoelectric focusing

The isoelectric point was determined by the method of Vesterberg and Svensson [28].

Amino acid analysis

Samples containing 3—5 mg inhibitor were hydrolyzed in vacuo at 110°C for 24, 48 and 72 h in 6 N HCl. HCl was removed under reduced pressure and the residue dissolved in 200 mM sodium citrate buffer (pH 2.2). Amino acid analyses were performed with 034 Hitachi Liquid Chromatograph. Tryptophan was determined by both the spectrophotometric method of Goodwin and Morton [29] and by amino acid analysis after hydrolysis in vacuo at 100°C for 42 h in 3 M *p*-toluenesulfonic acid containing 0.3% 3-(2-aminoethyl)indole [30]. Cysteine and cystine were determined as cysteic acid after oxidation with performic acid [31]. Free thiol content was determined by titration of the inhibitor with 5,5′-dithiobis(2-nitrobenzoic acid) in 50 mM Tris-HCl buffer (pH 8.0) [32].

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out in the absence or presence of sodium dodecyl sulfate on 15% polyacrylamide gels according to Davis [33] and Weber and Osborn [34], respectively. The reference proteins used for the molecular weight determination were ovalbumin (43 000), α-chymotrypsinogen (25 700), soybean trypsin inhibitor (20 000), cytochrome *c* (11 700) and insulin (6700).

Carbohydrate determination

The presence of carbohydrate was investigated by the phenol-H_2SO_4 [35] and Elson Morgan [36] methods.

Purification of Streptomyces metallo-proteinase inhibitor

Purification of the inhibitor was followed by inhibitory activity against *P. aeruginosa* IFO 3455 metallo-proteinase.

Step 1. Preparation of culture filtrate of *S. nigrescens* TK-23. After cultivation, the culture fluid was centrifuged at 6000 rev./min for to separate mycelia, and 8 l filtrate (containing 11 units inhibitor activity/ml) were obtained.

Step 2. Adsorption on Amberlite IRC-50. The pH of the filtrate was adjusted to 5.5 with glacial acetic acid. 800 ml Amberlite IRC-50 equilibrated with 20 mM sodium acetate buffer (pH 5.5) was added and the suspension was stirred for 2 h at room temperature. After checking the inhibitory activity, the resin was filtered on a Buchner funnel and washed thoroughly with tap water. The adsorbed inhibitor was then eluted for 30 min with 1 M sodium acetate solution adjusted to pH 10.5 with 5 M NaOH. The resin was filtered off and the pH of the eluate was immediately adjusted to 7. The eluate was concentrated in vacuo and then dialysed against distilled water, followed by buffer 1 (20 mM $Na_2CO_3/H_3BO_3/KCl$, pH 8.2).

Step 3. CM-Sephadex C-50 column chromatography. The dialyzed solution was applied to a CM-Sephadex C-50 column (5.5 × 40 cm) previously equilibrated with buffer 1. After washing the column with the same buffer, the inhibitor was eluted with a pH gradient (3 l equilibrating buffer and 3 l 100 mM $Na_2CO_3/H_3BO_3/KCl$ buffer, pH 10.5). The inhibitor was eluted at about pH 10 (Fig. 1). Fractions containing the inhibitor were pooled, and concentrated by ultrafiltration.

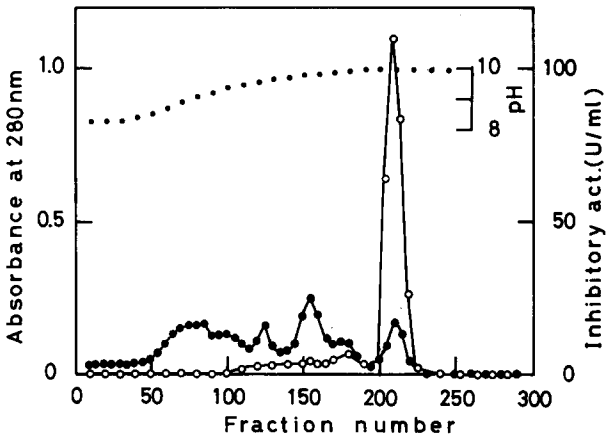

Fig. 1. Chromatography of the inhibitor on a CM-Sephadex C-50 column. The dialyzed solution containing inhibitor (total A_{280} = 7000; total I.U. = 71 300) in 2.5 l buffer 1 was applied to a column (5.5 X 40 cm) previously equilibrated with the same buffer. After washing the column with the same buffer, the inhibitor was eluted with a pH gradient of 3 l each of buffer 1 and 100 mM Na_2CO_3/H_3BO_3/KCl buffer (pH 10.5). Absorbance at 280 nm, ●———●. Inhibitory activity (unit/ml), ○———○. pH, ------.

Step 4. Sephadex G-50 column chromatography. The concentrated solution was applied to a Sephadex G-50 column (2.5 X 40 cm) previously equilibrated with buffer 1. The inhibitor was eluted with the same buffer. A single symmetrical peak with a constant specific activity of 800 inhibitor units/absorbance was found (Fig. 2). The fractions containing inhibitor were pooled and lyophilized, resulting in a white powder.

Results

A summary of the purification of inhibitor from a 10-l culture fluid of *S. nigrescens* TK-23 is given in Table I. The purified inhibitor represents a 2200-

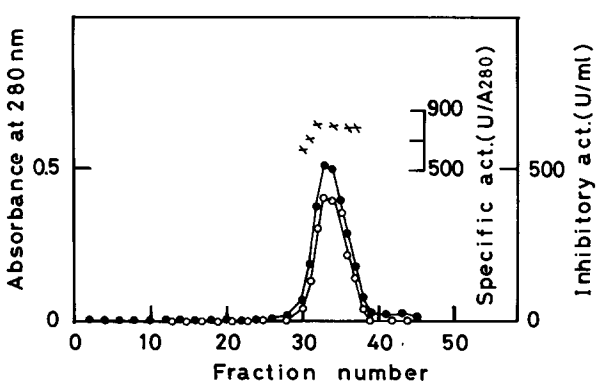

Fig. 2. Chromatography of the inhibitor on a Sephadex G-50 column. The partially purified inhibitor (total A_{280} = 14, total I.U. = 8900; spec. act. 636) was loaded on a column of Sephadex G-50 (2.5 X 40 cm) previously equilibrated with buffer 1. Absorbance at 280 nm, ●———●; inhibitory activity, ○———○; spec. act. (I.U./A_{280}), X.

TABLE I

SUMMARY OF PURIFICATION OF *STREPTOMYCES* PROTEINASE INHIBITOR

Procedure	Total protein ($A_{280} \times 10^{-3}$)		Total activity (Units $\times 10^{-3}$)	Spec. act. (Units/A_{280})	Yield (%)
Culture filtrate	8000	245	88.0	0.36	100
Amberlite IRC-50 (batch-wise)	2500	7	71.3	10.2	81
CM-Sephadex C-50 column chromatography	700	0.072	45.8	636	52
Sephadex G-50 column chromatography	92	0.043	35.2	819	40

fold purification over the original culture filtrate, with a 40% recovery (10 mg/l culture filtrate). The purified inhibitor displayed a single band when 10 μg protein were electrophoresed at pH 4.3, or at pH 7.0 with SDS after reduction and denaturation (Fig. 3).

Physicochemical properties

(a) Ultraviolet absorption spectrum. The ultraviolet absorption spectrum of the inhibitor in 50 mM Tris-HCl buffer (pH 7.5) showed maxima at 278 and

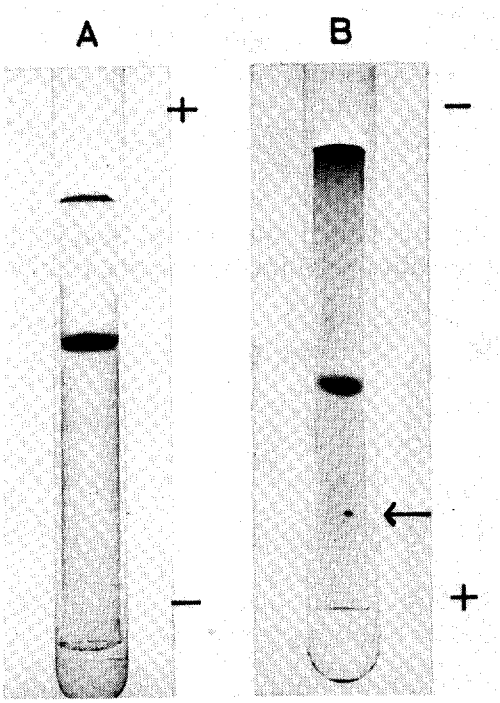

Fig. 3. Acrylamide gel electrophoresis of purified inhibitor. (a) Electrophoresis was carried out at pH 4.3 for 4 h on a 15% polyacrylamide gel column. (b) Electrophoresis was carried out at pH 7.0 with SDS, after reduction and denaturation, on a 15% polyacrylamide gel column (8 mA/tube). Protein (approx. 10 μg) was stained with Coomassie brilliant blue. Arrow indicates position of tracking dye.

285 nm, analogous to the typical absorption curve of tyrosine.

(b) Isoelectric point. Isoelectric focusing in an electrofocusing column containing ampholyte yielded a single peak, and its p*I* value was found to be 10.3.

(c) Stability. The inhibitor was heated at 100°C for 5 min in various buffers from pH 1.5 to 12. After cooling the remaining inhibitory activity was assayed. Between pH 2 and 7, the loss of activity was less than 20%. However, at pH 10, the inhibitory activity was completely destroyed.

(d) Amino acid composition. The amino acid composition is presented in Table II. Of particular interest is the absence of tryptophan residues. Sulfhydryl content was assayed with the Ellman reagent, 5,5′-dithiobis(2-nitrobenzoic acid); less than 0.03 mol thiol group/molecule was present. The absence of free thiol groups and presence of 4 mol half-cystine residues/molecule indicated the presence of two disulfide bridges/mol inhibitor. The molecular weight calculated on the basis of amino acid residues/molecule was 11 950.

(e) Carbohydrate content. Less than 0.05% carbohydrate was detected as glucose by using both the phenol-H_2SO_4 and Elson/Morgan methods. This indicated that the inhibitor is not a glycoprotein.

(f) Molecular weight. The molecular weight was estimated to be 12 000 by gel filtration on Sephadex G-75 equilibrated with 50 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl. The molecular weight was also estimated to be 12 000 by SDS-polyacrylamide gel electrophoresis. These values were very similar to those calculated from the amino acid composition.

(g) Inhibition spectrum. The inhibitory effect of the inhibitor on various proteinases including metallo-, serine-, thiol- and carboxyl-proteinases, was tested and the results are summarized in Table III. The inhibitor showed specific inhibitory activity for metallo-proteinases. Among the metallo-proteinases, thermolysin and proteinases of *P. aeruginosa* IFO 3455 and *B. subtilis* YT-25 were strongly inhibited, whereas the metallo-proteinases of *B. subtillis* var. *amylosaccharitícus*, *B. amyloliquefaciens*, and *Serratia sp.* E-15

TABLE II

AMINO ACID COMPOSITION OF *STREPTOMYCES* PROTEINASE INHIBITOR

Amino acid	Residues/molecule [a]	Amino acid	Residues/molecule [a]
Lys [b]	5	Ala [b]	17
His [b]	1	Val [b]	10
Arg [b]	8	Met [b]	1
$Cys_{1/2}$ [e]	4	Ile [b]	1
Asp [b]	6	Leu [b]	8
Thr [c]	13	Tyr [b]	4
Ser [c]	11	Phe [b]	3
Glu [b]	5	Try [d]	0
Pro [b]	5		
Gly [b]	17	Total	119

[a] Based on a molecular weight of 12 000.
[b] Average values from 24-, 48- and 72-h hydrolysates.
[c] Values extrapolated to zero hydrolysis time.
[d] Determined spectrophotometrically by the method of Goodwin and Morton [29].
[e] Determined as cysteic acid on a 24-h hydrolysate after performic acid oxidation.

TABLE III

INHIBITORY EFFECT OF THE INHIBITOR ON VARIOUS PROTEINASES

Proteinases	Amount of enzyme (μg)	Substrate	μg needed for 50% inhibition
Metallo-proteinases			
Thermolysin	10	casein	3.0
	1	Z-Gly-Leu · NH_2	0.36
P. aeruginosa neutral proteinase	10	casein	1.8
	1	Z-Gly-Leu · NH_2	0.17
B. subtilis YT-25 neutral proteinase	10	casein	1.8
	1	Z-Gly-Leu · NH_2	0.2
B. subtilis var.	10	casein	÷200
amylosaccharaticus neutral proteinase	1	Z-Gly-Leu · NH_2	÷100
B. amyloliquefaciens neutral proteinase	10	casein	÷200
	1	Z-Gly-Leu · NH_2	÷100
Serratia sp. proteinase	10	casein	÷200
	1	Z-Gly-Leu · NH_2	÷100
Bovine carboxypeptidase A	11	Hippuryl-L-Phe	>500
Pig leucine aminopeptidase	92	L-leucine amide	>100
C. histolyticum collagenase	6	Azocoll	1.5
Serine proteinases			
Bovine trypsin	10	casein	>100
Bovine chymotrypsin	10	casein	>100
Bovine pancreatic kallikrein	38	Bz-Arg-OEt	>100
Bovine pancreatic elastase	14	Boc-Ala-ONp	>100
Human pancreatic elastase	500	Boc-Ala-ONp	>100
Subtilisin BPN'	25	casein	>100
Thiol proteinases			
Papain	25	casein	>100
Ficin	100	casein	>100
Carboxyl proteinases			
Porcine pepsin	25	casein	>100
Rhodotorula glutinis acid proteinase	25	casein	>100
Cladosporium sp. acid proteinase	25	casein	>100
Rhizopus chinesis acid proteinase	25	casein	>100
Scytalidum lignicolum			
acid proteinase A	50	casein	>100
acid proteinase B	50	casein	>100

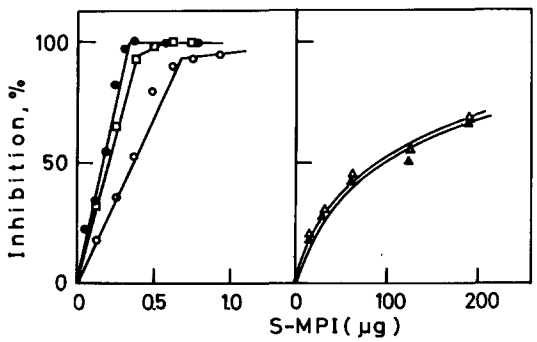

Fig. 4. Inhibitory effect on various metallo-proteinases. The reaction mixture consisted of 2 μmol Z-Gly-Leu · NH_2, 1 μg enzyme, and various amounts of inhibitor in 1 ml 10 mM Tris-HCl buffer (pH 7.0), 10 mM $CaCl_2$. After reaction at 37°C for 20 min, the residual activities were assayed with ninhydrin. *P. aeruginosa* metallo-proteinase, ●———●; *B. subtilis* YT-25 metallo-proteinase, □———□; thermolysin, ○———○; *B. amyloliquefaciens* neutral proteinase, △———△; and *B. subtilis* var. *amylosaccharitiicus* neutral proteinase, ▲———▲.

were slightly inhibited (Fig. 4). Carboxypeptidase A and leucine aminopeptidase were not inhibited.

Discussion

The inhibitor was isolated as an electrophoretically homogeneous preparation from the culture filtrate of *S. nigrescens* TK-23. The inhibitor is a simple and heat-stable protein with a molecular weight of approx. 12 000. With regard to amino acid composition, the high content of hydrophobic amino acids and the lack of tryptophan were of particular interest. Two disulfide bridges were found. With regard to the inhibition spectrum against various proteinases, the inhibitor showed a specific inhibitory activity only against the metallo-proteinases but not serine-, thiol- or carboxyl-proteinases. Among the metallo-proteinases inhibited, there existed two groups on the basis of the amount of inhibitor necessary for 50% inhibition of activity, i.e., those of one group form strong complexes with low dissociation constants (thermolysin, *P. aeruginosa* metallo-proteinase), whereas those of the other group form weak, highly dissociable complexes (*B. amyloliquefaciens* metallo-proteinase, *B. subtilis* var. *amylosacchariticus* metallo-proteinase). These differences in degree of inhibition might reflect the difference of their substrate specificities. In fact, carboxypeptidase A and leucine aminopeptidase were not inhibited.

It was estimated that the inhibitor reacts with enzyme in a 1 : 1 molar ratio as judged by enzymic titration of inhibitor, molecular weight determination of enzyme-inhibitor complex, and measurement of differences spectra in the ultraviolet range caused by complex formation (unpublished data).

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