J. Enzyme Inhibition, 1996, Vol. 10, pp. 93–103
Reprints available directly from the publisher
Photocopying permitted by license only

PIPERASTATIN A, A NEW SELECTIVE SERINE CARBOXYPEPTIDASE INHIBITOR PRODUCED BY ACTINOMYCETE. I. TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL ACTIVITIES

SHOICHI MURAKAMI,[1] SHIGEKO HARADA,[1] TAKAKO YAMAZAKI,[1] YOSHIKAZU TAKAHASHI,[1] MASA HAMADA,[1] TOMIO TAKEUCHI[1] and TAKAAKI AOYAGI[1,2,†]

[1]*Institute of Microbial Chemistry, 3–14–23 Kamiosaki, Sinagawa-ku, Tokyo 141, Japan*

[2]*Showa College of Pharmaceutical Sciences, 3–3165 Higashitamagawagakuen, Machida-shi, Tokyo 194, Japan*

(Received 24 July 1995)

Piperastatin A (structure, N-formyl-*allo* Ile-Thr-Leu-Val-Pip-Leu-Pip, Pip = hexahydropyridadine-3-carboxylic acid; molecular weight, 809), a new inhibitor of serine carboxypeptidase was discovered in the fermentation broth of *Streptomyces lavendofoliae* MJ908-WF13. It was purified by activated charcoal chromatography, YMC gel ODS-A chromatograpy and centrifugal partition chromatography (CPC) by monitoring its inhibitory activity against carboxypeptidase Y (CP-Y), and finally obtained as colourless needles. Piperastatin A is a competitive inhibitor of the enzyme with $K_i = 52 \pm 6.2$ nM. Piperastatin A is a highly specific inhibitor of the serine carboxypeptidases, CP-Y and platelet deamidase with little effect on related enzymes, has no antimicrobial activity and has low toxicity.

KEY WORDS: Serine carboxypeptidase, carboxypeptidase Y, enzyme inhibitor, peptide, Actinomycete, natural compound

INTRODUCTION

Serine carboxypeptidases are widely distributed in higher organisms and have different tissue specificities and substrate specificities[1] when compared with metallo carboxypeptidases, such as carboxypeptidase A (CP-A), CP-B[2] or CP-N.[3] Carboxypeptidase Y[1] (CP-Y), gene product of KEX1 of yeast,[4] CP-W of wheat[5] or platelet deamidase of human[6] are also serine carboxypeptidases. In the case of mammalian organs, platelet deamidase (identified

†Correspondence.

Abbreviations: Boc, *t*-butyloxycarbonyl; Bz, benzoyl; Suc, succinyl; Z, carbobenzoxy; βNA, β-naphthylamide; CPC, centrifugal partition chromatography; CPase, carboxypeptidase; MGPA, D,L-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid; MW, molecular weight; S.E., standard error of mean.

as protective protein) might inactivate tachykinins such as substance P or neurokinin A by deamidating their protected carboxyl termini, and it was recently reported that CP-Y like kininase contributed to the degradation of bradykinin in rat urine[7] and might play an important role in the hypertensive pathway.[8] In order to understand the physiological roles of these enzymes or to find a useful tool for biochemical, cell biological or pharmaceutical studies on them, we have screened for inhibitors in microbial products[9] and discovered a new inhibitor against CP-Y in the culture broth from *Streptomyces lavendofoliae* MJ908-WF13.

In this communication we report the taxonomy, production, isolation, and enzymatic inhibitory activities and other biological activities of this new serine carboxypeptidase inhibitor, piperastatin A, obtained from Actinomycete.

MATERIALS AND METHODS

Microorganism and Taxonomic Characterization

Strain MJ908-WF13 was isolated from a soil sample collected in Niiza-shi, Saitama, Japan and has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan, under the accession No. FERM P-13940.

Morphological and physiological characteristics of strain MJ908-WF13 were examined according to the methods described by Shirling and Gottlieb,[10] and Waksman.[11] Cultures were incubated at 27°C for 2 to 4 weeks and colour descriptions were made using colour chips from Color Harmony Manual (Container Corporation of America).

Chemicals

Chemicals employed were as follows: Activated carbon from Wako Pure Chemical Industries Ltd., Osaka, Japan; TLC-plate Silica gel F254 (0.25 mm thickness) from E. Merck, Darmstadt, FRG; DEAE sephadex A-50 from Pharmacia Fine Chemicals AB, Uppsala, Sweden; Capcellpak C18 S-5 μm reversed-phase column (4.6 × 150 mm) from Shiseido Co., Tokyo, Japan; YMC gel ODS-A from Yamamura Chemical Laboratories Co. Ltd., Kyoto, Japan; *p*-nitrophenyl acetate (PNP acetate), benzoyl-glycyl-L-phenylalanine (Bz-Gly-Phe), L-leucine-β-naphthylamide (Leu-βNA) from Sigma Chem. Ltd., Saint Louis, USA; benzoyl-glycyl-L-lysine (Bz-Gly-Lys), benzoyl-glycyl-L-histidyl-L-leucine (Bz-Gly-His-Leu), *t*-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine 4-methyl-coumaryl-7-amide (Boc-V-P-R-MCA), succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methyl-coumaryl-7-amide (Suc-L-L-V-Y-MCA), D-Ala^2-Met^5- enkephalin, D-Ala^2-Met^5-enkephalinamide from Peptide Institute Inc., Minoh-shi, Japan; glycyl-L-arginine-β-naphthylamide (Gly-Arg-βNA), L-lysyl-L-alanine-β-naphthylamide (Lys-Ala-βNA), glycyl-L-proline-β-naphthylamide (Gly-Pro-βNA), carbobenzoxy-L-arginyl-L-arginine-β-naphthylamide (Z-Arg-Arg-βNA), benzoyl-L-phenylalanyl-L-valyl-L-arginine-4-methoxy-β-naphthylamide (Bz-Phe-Val-Arg-4-methoxy-βNA), carbobenzoxy-glycyl-L-proline-β-naphthylamide (Z-Gly-Pro-βNA) from Bachem Freinchemikalien AG, Bubbendorf, Switzerland; MGPA from Calbiochem Co., La Jolla, USA. All other chemicals were of analytical grade.

Enzymes

Carboxypeptidase Y (EC 3.4.16.1) from yeast was obtained from Oriental Yeast Co. Ltd., Japan. Carboxypeptidase A (CP-A) type I from bovine pancreas, thrombin from human plasma, α-chymotrypsin type II from bovine pancreas and cathepsin B from bovine spleen were purchased from Sigma Chem. Ltd., Saint Louis, USA. Carboxypeptidase B (CP-B) from porcine pancreas, aminopeptidase N (AP-N) from hog kidney, leucine aminopeptidase (Leu-AP) from hog kidney and esterase from hog liver were purchased from Boehlinger Mannheim GmbH, FRG. Lipase from hog pancreas was obtained from Nutritional Biochem Co., USA. Carboxypeptidase N (CP-N) from human plasma, dipeptidylaminopeptidase I (DPP-I) from rat spleen, DPP-II from rat spleen, DPP-IV from rat kidney and prolylendopeptidase (PEP) from hog kidney were partially purified as follows.[12] CP-N was prepared from human plasma by ammonium sulfate precipitation (40 $\sim$ 80%) and DEAE Sephadex A-50. DPP-I and DPP-II were prepared from rat spleen homogenate by ammonium sulfate precipitation (40 $\sim$ 80%). DPP-IV was prepared from rat kidney homogenate by ammonium sulfate precipitation (30 $\sim$ 80%). PEP was prepared from hog kidney by ammonium sulfate precipitation (50 $\sim$ 65%) and DEAE Sephadex A-50. Angiotensin converting enzyme (ACE) was prepared from bovine lung homogenates by solubilizing with Triton X-100 (0.1%, W/V). Platelet deamidase was prepared as a released product from human platelets, which were activated with epinephrine, thrombin and $CaCl_2$ as described by Jackman *et al.*[6] Washed platelets were purchased from healthy volunteers according to the partially modified method by Yamamoto *et al.*[13]

Production of Piperastatin A

Strain MJ908-WF13 was inoculated into a 500 ml Erlenmeyer flask containing 110 ml of a medium consisting of glucose 2.0% (W/V), glycerin 2.0%, Pharma media 1.0%, NaCl 0.5%, soy bean meal 1.2%, $CaCO_3$ 0.32% and $MnCl_2 \cdot 4H_2O$ 0.005% (pH 7.4) and cultured at 27°C for 2 or 3 days on a rotary shaker (180 rpm). Two ml of the above seed culture was transferred to 110 ml of the same medium in a 500 ml Erlenmeyer flask and cultured at 30°C for 112 h. The process of production of piperastatin A was followed by the inhibitory activity of 2 μl of broth filtrate against CP-Y (assay volume, 100 μl). The time course of the production of piperastatin A is shown in Figure 1.

Isolation of Piperastatin A

The purification procedure for piperastatin A is shown in Figure 2. The inoculated medium was incubated at 30°C for 112 hours and then the cultured broth was separated from mycelium cake by filtration. The culture filtrate (10 liters) was applied to an activated carbon column (1 liter). The column was washed with water and was eluted with 70% aqueous 1-propanol (pH 7.0). The active fractions were pooled and concentrated under reduced pressure to give a crude powder (23.4 g). The crude powder was dissolved in water, subjected to a YMC-gel ODS-A column (250 ml) and eluted with a linear gradient from 20% to 100% MeOH. The active fractions were pooled, concentrated under reduced pressure and lyophilized to give a brownish powder (414 mg).

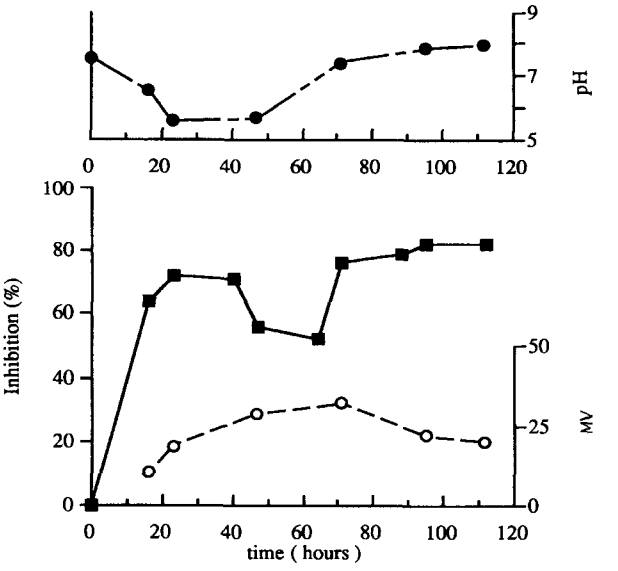

FIGURE 1 Time course of CP-Y inhibitor production by *Streptomyces lavendofoliae* MJ908-WF13: (■) Inhibition (%) of broth filtrate (20 μl/ml), (○) mycelial volume (MV, %), (●) pH.

Streptomyces lavendofoliae MJ908-WF13

30℃, 112hrs.

Culture filtrate (10L, IC_{50} = 3.4 μ l/ml)

Activated carbon chromatography

70% 1-propanol

YMC gel ODS chromatography

linear gradient 20 to 100% MeOH

Centrifugal partition chromatography (three times)

$CHCl_3$-MeOH-H_2O (5:6:4)

Crystallization from MeOH-H_2O

Piperastatin A (colorless needles) , 17mg

IC_{50}=0.052 μ g/ml

FIGURE 2 Isolation procedure for piperastatin A.

TABLE 1
Comparison of characteristics of strain MJ908-WF13 with *S. lavendofoliae* IMC S-0784 and *S. lavendofoliae* IMC S-0213.

	MJ908-WF13	*Streptomyces lavendofoliae* IMC S-0784 (MI951–62F2)	*Streptomyces lavendofoliae* IMC S-0213^T (ISP 5217)
Spore chain morphology	spirals	spirals	spirals
Spore surface	smooth	smooth	smooth
Spore size (μm)	0.5~0.9×0.7~1.0	0.6~0.9×0.3~0.6	0.6~0.9×0.5~0.8
Aerial mass color	pale pink ~ pinkish gray	pale pink ~ pinkish gray	pale pink
Vegetative growth color	pale yellow ~ pale yellowish brown	pale yellow	pale yellow ~ yellowish brown
Soluble pigments	faint brownish	faint brownish	faint brownish
Melannoid pigments formation			
ISP1	+	+	+
ISP6	+	+	+
ISP7	+	+	+
Liquefaction of gelatin	+ (weak)	–	+ (weak)
Coagulation of skim milk	+ or –	+ or –	+ or –
Peptonization of skim milk	+ (weak)	+ (weak)	+
Nitrate reduction	+	+	–
Hydrolysis of starch	+	+	+
Carbon utilization**			
L-Arabinose	+	+	+
D-Xylose	+	+	+
D-Fructose	–	–	–
D-Glucose	+	+	+
Rhamnose	(–)	(–)	–
Sucrose	–	–	–
Raffinose	–	–	–
Inositol	–	–	+
D-Mannitol	–	–	–

** +: utilization; (–): probably no utilization; –: no utilization; T: standard strain.

The crude powder was subjected to centrifugal partition chromatography (CPC). The chromatography was performed using a CPC apparatus model NMF (Sanki Engineering Ltd.) under the following conditions: solvent system, $CHCl_3$-MeOH-H_2O (5:6:4); ascending mode; flow rate, 5 ml/min; centrifugation, 700 rpm; temperature, 20°C. After repeating CPC three times under the same conditions, fractions including pure inhibitor were collected

TABLE 2
Conditions for enzyme assays.

Enzyme	Substrate	Reaction media
CP-Y	1 mM Bz-Gly-Phe	25 mM Na phosphate buffer (pH 6.5)
CP-A	1 mM Bz-Gly-Phe	25 mM Na phosphate buffer (pH 7.5) containing 0.9 M NaCl
CP-B	1 mM Bz-Gly-Lys	25 mM Tris-HCl buffer (pH 8.0)
CP-N	1 mM Bz-Gly-Lys	25 mM Tris-HCl buffer (pH 7.2)
ACE	1.2 mM Bz-Gly-His-Leu	50 mM Tris-HCl buffer (pH 8.0) containing 30 mM NaCl
Leu-AP	0.5 mM Leu-βNA	50 mM Tris-HCl buffer (pH 7.5)
AP-N	0.5 mM Leu-βNA	50 mM Tris-HCl buffer (pH 7.0)
DPP-I	0.4 mM Gly-Arg-βNA	50 mM Citrate buffer (pH 4.0) containing 5 mM NaCl, 7 mM 2-mercaptoethanol
DPP-II	0.4 mM Lys-Ala-βNA	50 mM 3,3-dimethylglutalic acid-NaOH buffer (pH 5.5)
DPP-IV	0.5 mM Gly-Pro-βNA	50 mM Tris-Malate-NaOH buffer (pH 7.0)
Chymotrypsin	0.2 mM Suc-L-L-V-Y-MCA	50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 1 mM $CaCl_2$
Thrombin	0.2 mM Boc-V-P-R-MCA	50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl
PEP	0.1 mM Z-Gly-Pro-βNA	25 mM Tris-HCl (buffer (pH 7.8)
Esterase	0.125 mM PNP acetate	50 mM Na phosphate buffer (pH 7.0) containing 0.03% triton X-100
Lipase	0.125 mM PNP acetate	50 mM Na phosphate buffer (pH 7.0) containing 0.03% triton X-100

and concentrated to a small volume and were lyophilized to give a colourless powder (35 mg). This was crystallized from MeOH-H_2O to give colourless needles (17 mg).

Enzyme Assays

The assays for some of the enzymes described here were performed according to the previously reported methods.[12] The remaining enzyme assays are described below, and the conditions for all the enzyme assays are summarized in Table 2.

The principle of the assays for carboxypeptidases and ACE activities is based on the spectrometric determination of N-benzoylglycine with cyanuric chloride[13] using a microplate reader model 3550 (BIO-RAD). The reaction mixture (total 0.1 ml) for CP-Y consisted of 25 mM sodium phosphate buffer (pH 6.5), 1 mM Bz-Gly-Phe, 2.0 μg/ml enzyme, and water or aqueous solution containing the test compound. After incubation at 37°C for 40 min, 6 μl of 1 N sodium hydroxide, 50 μl of 0.36 M sodium phosphate buffer (pH 7.2) and 0.15 ml of 2% (W/V) cyanuric chloride, dissolved in 2-methoxyethanol were successively added to the mixed solution. CP-Y activity was determined by measuring the absorbance at 405 nm.

For aminopeptidases, dipeptidylaminopeptidases, cathepsin B and prolylendopeptidase, β-naphthylamide derivatives were used as the substrates and the activities of enzymes were determined from the absorbance at 525 nm of β-naphthylamine liberated in these assay systems.[14] The activities of lipase and esterase were determined from the absorbance at 405 nm of *p*-nitrophenol liberated in these enzyme reactions.[15]

The principle of the assays for chymotrypsin and thrombin is based on the fluorescence measurement of 7-amino-4-methylcoumarin (AMC) released from the fluorogenic substrates used[16] by the Cytofluor 2350 Fluorescence Measurement System (Millipore Co.). In the assay of thrombin, the reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 0.2 mM Boc-Val-Pro-Arg-MCA, 50 U/ml enzyme, and water or aqueous solution containing the test compound. The enzyme reaction was started by the addition of the enzyme, followed by incubation at 37°C for 30 min. The reaction was stopped by adding 25 μl of 10% SDS. After the mixture was added with 25 μl of 1 M Tris-HCl buffer (pH 9.0) the enzymatic activity was determined by measuring the fluorescence (excitation, 360 nm; emission, 460 nm).

The deamidase activity was determined by measuring the appearance of D-Ala^2-Met^5-enkephalin, which is the deamidated product from D-Ala^2-Met^5-enkephalinamide according to the partially modified method by Jackman *et al.*[6] The reaction mixture contained 100 mM HEPES buffer (pH 7.0), 17.6 μM D-Ala^2-Met^5-enkephalinamide, 5 μl enzyme fraction, and water or aqueous solution containing the test compound in a final volume of 0.1 ml. After incubation at 37°C for 30 min, the reaction was terminated by addition of 0.1 ml of 1% trifluoroacetic acid (TFA) aqueous solution and then 50 μl of this solution was subjected to HPLC analysis to determine the extent of the hydrolysis.

For HPLC analysis, GILSON's system (Gilson Medical Electronics Inc., Middleton, USA) was used, consisting of a model 305/306 pump system, a model 231–401 autosampler and a model 116 detecter, and the substrate and product of the hydrolysis were separated on a Shiseido capcellpak C18 S-5 μm reversed-phase column (4.6 × 150 mm), which was eluted with 0.1% TFA aqueous solution including 20% acetonitrile at a flow rate of 1.0 ml/min. The chromatography was monitored at 210 nm and the peaks were quantified by integration and comparison to known quantities of authentic standards.

The percent inhibitions were calculated by the formula (A-B)/A × 100, where A is the value obtained in the enzymatic assay without an inhibitor and B is that with an inhibitor. The IC_{50} value is the concentration of inhibitor giving 50% inhibition of enzyme activity.

RESULTS AND DISCUSSION

Taxonomy of the Producing Organism

Taxonomic features of strain MJ908-WF13 are shown in Table 1. Strain MJ908-WF13 has branched substrate mycelia developing spiral aerial mycelia and no whirl-formation, or sporangia were observed. Matured spore-chains usually bear more than 50 cylindrical spores and the spores ranged about 0.5 ~ 0.9 by 0.7 ~ 1.0 μm in size and had a smooth surface. The colour of the vegetative growth was pale yellow to pale yellowish brown, the aerial mass colour of the colony was pale pink to pinkish gray and the production of melanoid pigments was positive.

The whole cell hydrolysate of the strain showed that it contained LL-diaminopimelic acid. Based on its characteristics, strain MJ908-WF13 was considered to belong to the genus *Streptomyces*. As shown in Table 1, the taxonomic features of *S. lavendofoliae* ISP 5217[18] are very similar to those of the strain MJ908-WF13 except for nitrate reduction and inositol utilization, and the taxonomic features of this strain are also similar to those of *S. lavendofoliae* MI951–62F2,[19] which is the producer of depsidomycin, except for liquefaction of gelatin. Therefore, we estimated that the strain MJ908-WF13 belonged to *Streptomyces lavendofoliae,* and it was designated as *Streptomyces lavendofoliae* MJ908-WF13.

Production and Isolation of Piperastatin A

The strain of *Streptomyces lavendofoliae* MJ908-WF13 was cultured in Erlenmeyer flasks at 30°C for 6 days on a rotary shaker. The time course of the production for 112 h is shown in Figure 1. The second maximum peak of production of inhibitor was obtained after 6 days or later. As shown in Figure 2, a major component, piperastatin A was isolated by several purification steps and obtained as colourless needles (17 mg) from the culture filtrate (10 liters). A minor component was separated from piperastatin A by CPC, and its further purification and characterization remains to be performed. The purity of piperastatin A was confirmed by silica gel TLC ($CHCl_3$-MeOH-H_2O, 65:25:2, Rf = 0.40) and HPLC under the following conditions: column, Capcellpak C18 S-5 μm (4.6 × 150 mm, Shiseido Co.); flow rate, 1.0 ml/min; gradient, 20% to 50% aqueous acetonitrile for 25 min; detection, UV at 210 nm. The physico-chemical properties and determination of the structure of piperastatin A are reported in the following paper.[20]

Enzymatic Inhibitory Activities or Other Biological Activities of Piperastatin A

The inhibitory activity (IC_{50}) of piperastatin A against carboxypeptidase Y was determined to be 52 ng/ml (64 nM; MW, 809). The Lineweaver-Burk plot of piperastatin A against CP-Y exhibited competitive inhibition as shown in Figure 3. The K_i value of piperastatin A was determined to be 52 ± 6.2 nM (mean ± S.E., $n = 3$) by Dixon plots of CP-Y inhibition (not shown). Piperastatin A had no antimicrobial activity at 100 μg/ml, though depsidomycin, a structurally related depsipeptide had antimicrobial activity against Gram-positive microorganisms.[19] It has low toxicity; there were no deaths after intraperitoneal injection of mice with 100 mg/kg of piperastatin A (data not shown).

In previous work we discovered belactins, new serine carboxypeptidase inhibitors, possessing a β-lactone moiety with specific inhibitory activities against CP-Y.[12,21] In this work piperastatin A was considered to have high selectivity towards CP-Y among many kinds of enzymes; metallo carboxypeptidases (CP-A, B and N), dipeptidylcarboxypeptidase (ACE), aminopeptidases (AP-M and Leu-AP), dipeptidylaminopeptidases (DPP-I, II and IV), cysteine proteinase (cathepsin B), serine proteinases (chymotrypsin, thrombin and PEP), lipase and esterase except that it inhibited ACE weakly (Table 3). Piperastatin A, the structure of which was determined to be N-formyl-*allo* Ile-Thr-Leu-Val-Pip-Leu-Pip (pip, piperazic acid = hexahydropyridadine-3-carboxylic acid) as reported in the following paper,[20] is a new type of selective serine carboxypeptidase inhibitor.

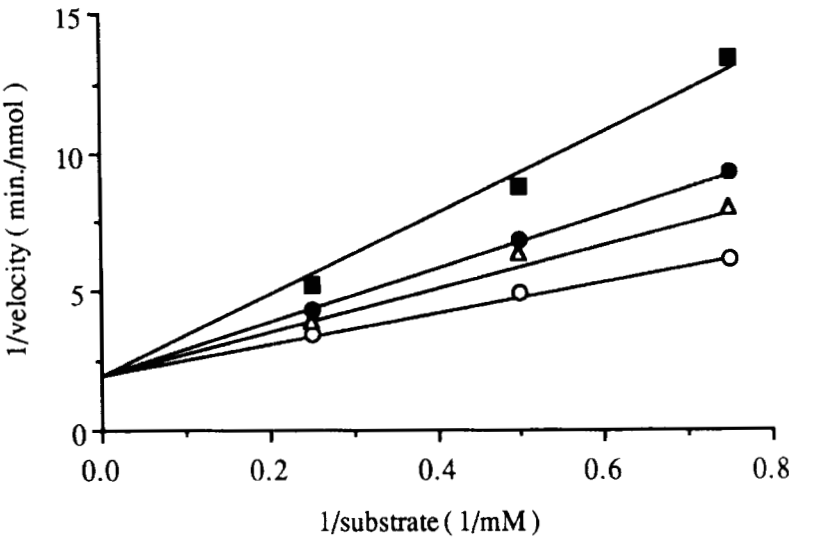

FIGURE 3 Lineweaver-Burk plot of inhibition of CP-Y by piperastatin A. (○) I = 0 μM; (△) I = 0.043 μM; (●) I = 0.065 μM; (■) I = 0.087 μM. I = inhibitor.

The effects of piperastatin A and other carboxypeptidase inhibitors on various carboxypeptidases were examined and the results are shown in Table 4. Benzylmalic acid,[22] histargin[23,24] or MGPA,[25] which are known to be metallo carboxypeptidase inhibitors, did not inhibit serine carboxypeptidases, but piperastatin A specifically inhibited CP-Y and deamidase from human platelet. Belactins A and B, which we have discovered as CP-Y inhibitors, inhibited CP-Y more specifically, and the differential inhibitory activity of piperastatin A and belactins against CP-Y and platelet deamidase is helpful in applying serine carboxypeptidase inhibitors for many purposes. In previous studies, DFP (diisopropylfluorophosphate),[1] or acylaminoacids[26] were found to be inhibitors of CP-Y, and platelet deamidase was reported to be inhibited by DFP, CK8 (Z-Gly-Leu-Phe-CH_2Cl), PCMS (*p*-chloromercuriphenylsulfonate), $HgCl_2$ or chymostatin, a chymotrypsin inhibitor.[27] Recently Majima *et al.* detected a kininase activity which cleaved carboxyterminal arginine (Arg^9) from bradykinin in rat ureters urine and found that the enzyme activity, which was designated CP-Y like kininase, was inhibited by poststatin[27] (an inhibitor of PEP) and ebelactone B^8 (an inhibitor of lipase) and suggested that the degradation of bradykinin by this enzyme might be responsible for the development of hypertension. In this context piperastatin A has a highly selectivity against serine carboxypeptidases, such as CP-Y or platelet deamidase and may be a useful tool for understanding the physiological role of serine carboxypeptidases such as platelet deamidase or CP-Y like kininase.

TABLE 3
Inhibitory activities of piperastatin A against various enzymes.

Enzymes	Origin	IC_{50} (μg/ml)
CP-Y	yeast	0.052
CP-A	bovine pancreas	>100
CP-B	porcine pancreas	>100
CP-N	human plasma	>100
ACE	bovine lung	34
Leu-AP	hog kidney	>100
AP-N	hog kidney	>100
DPP-I	rat spleen	>100
DPP-II	rat spleen	>100
DPP-IV	rat kidney	>100
Chymotrypsin	bovine pancreas	>100
Thrombin	human plasma	>100
PEP	hog kidney	>100
Cathepsin B	bovine spleen	>100
Lipase	hog pancreas	>100
Esterase	hog liver	>100

TABLE 4
Inhibitory activities of various inhibitors against carboxypeptidases.

	IC_{50} (μg/ml)				
	Metallo-CPases			Serine-CPases	
	CP-A	CP-B	CP-N	CP-Y	Deamidase
Piperastatin A	>100	>100	>100	0.052	8.4
Belactin A	>100	>100	>100	0.060*	>100
Belactin B	>100	>100	>100	0.14*	>100
Benzylmalic acid	0.40	10	>100	>100	>100
Histargin	>100	17	6.0	>100	>100
MGPA	>100	0.4	0.02	>100	>100

*preincubation of inhibitors with the enzyme at 37°C for 40 min was performed.

References

1. Hayashi, R., Bai, Y. and Hata, T. (1974) *J. Biochem.*, **76**, 1355.
2. Sokolovsky, M. (1977) In *Meth. Enzymol.*, **46** pp. 225, New York; Academic Press.
3. Plummer, T., Jr. and Erdos, E.G. (1981) In *Meth. Enzymol.*, **80**, pp. 442. New York; Academic Press.

4. Dmochowska, A., Dignard, D., Henning, D., Thomas, D.Y. and Bussey, H. (1987) *Cell*, **50**, 573.
5. Breddam, K., Sorenson, S.B. and Svendsen, I. (1987) *Carlsberg Res. Commun.*, **52**, 297.
6. Jackman, H.L., Tan, F.L., Tamei, H., Beurling, H.C., Li, X.Y., Skidgel, R.A. and Erdos, E.G. (1990) *J. Biol. Chem.*, **265**, 11265.
7. Kuribayashi, Y., Majima, M., Katori, M. and Kato, H. (1993) *Biomedical Res.*, **14**, 191.
8. Majima, M., Shima, C., Saito, M., Kuribayashi, Y., Katori, M. and Aoyagi, T. (1993) *Eur. J. Pharmacol.*, **232**, 181.
9. Aoyagi, T. (1990) In *Biochemistry of Peptide Antibiotics-Recent advances in the biotechnology of β-lactams and microbial bioactive peptides* (Kleinkauf, H. and Dohren, H. von, ed.) pp. 311. New York; Walter de Gruyter Berlin.
10. Shirling, E.B. and Gottlieb, D. (1966) *Int. J. Syst. Bacteriol.*, **16**, 313.
11. Waksman, S.A. (1961) In *The Actinomycetes*, **2**, pp. 1. Baltimore; The Williams & Wilkins Co.
12. Murakami, S., Harada, S., Kojima, F., Kinoshita, N., Takahashi, Y., Hamada, M., Takeuchi, T. and Aoyagi, T. (1995) *J. Enz. Inhib.*, in press.
13. Hayakari, M., Kondo, Y. and Izumi, H. (1978) *Anal. Biochem.*, **84**, 361.
14. Aoyagi, T., Wada, T., Kojima, F., Nagai, M. and Umezawa, H. (1981) *J. Clin. Invest.*, **67**, 51.
15. Kawabata, S., Miura, T., Morita, T., Kato, H., Fujikawa, K., Iwanaga, S., Takada, K., Kimura, T. and Sakakibara, S. (1989) *Eur. J. Biochem.*, **172**, 17.
16. Umezawa, H., Aoyagi, T., Hazato, T., Uotani, K., Kojima, F., Hamada, M. and Takeuchi, T. (1978) *J. Antibiot.*, **31**, 639.
17. Yamamoto, N., Kitagawa, H., Yamamoto, K., Tanoue, K. and Yamazaki, H. (1989) *Blood*, **73**, 1552.
18. Locci, R. (1989) In *Bergey's Manual of Systematic Bacteriology* (S.T. Williams, M.E.S. and J.G.H, ed.) **4**, pp. 2490. Baltimore; The Williams & Wilkins Co.
19. Isshiki, K., Sawa, T., Naganawa, H., Koizumi, Y., Masuda, N., Hamada, M., Takeuchi, T., Iijima, M., Osono, M., Masuda, T. and Ishizuka, M. (1990) *J. Antibiot.*, **43**, 1195.
20. Murakami, S., Takahashi, Y., Naganawa, H., Takeuchi, T. and Aoyagi, T. (1995) *J. Enz. Inhib.*, in press.
21. Murakami, S., Takahashi, Y., Naganawa, H., Takeuchi, T. and Aoyagi, T. (1995) *J. Enz. Inhib.*, in press.
22. Tanaka, T., Suda, H., Naganawa, H., Hamada, M., Takeuchi, T., Aoyagi, T. and Umezawa, H. (1984) *J. Antibiot.*, **37**, 682.
23. Umezawa, H., Aoyagi, T., Ogawa, K., Iinuma, H., Naganawa, H., Hamada, M. and Takeuchi, T. (1984) *J. Antibiot.*, **37**, 1088.
24. Ogawa, K., Naganawa, H., Iinuma, H., Aoyagi, T. and Umezawa, H. (1984) *J. Antibiot.*, **37**, 984.
25. Plummer, T.H. and Ryan, T.J. (1981) *Biochem. Biophys. Res. Commun.*, **98**, 448.
26. Hayashi, R. (1976) In *Meth. Enzymol.*, (Lorand, L., ed.) **45**, pp. 568. New York; Academic Press.
27. Majima, M., Kuribayashi, Y., Ikeda, Y., Adachi, K., Kato, H., Katori, M. and Aoyagi, T. (1994) *Jpn. J. Pharmacol.*, **65**, 79.