POSTSTATIN, A NEW INHIBITOR OF PROLYL ENDOPEPTIDASE, PRODUCED BY *Streptomyces viridochromogenes* MH534-30F3

I. TAXONOMY, PRODUCTION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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Poststatin, a new inhibitor of prolyl endopeptidase (PEP) was discovered in the fermentation broth of *Streptomyces viridochromogenes* MH534-30F3. It was purified by Diaion HP-20, Sephadex LH-20 and YMC-gel (ODS-A) column chromatography and then isolated as a colorless powder. Poststatin has the molecular formula $C_{26}H_{47}N_5O_7$. The IC_{50} value of poststatin against the PEP of partially purified porcine kidney was 0.03 μg/ml. It has low acute toxicity. No deaths occured after *iv* injection of 250 mg/kg of this agent to mice.

We have been studying enzyme networks in various human and animal models of disease, including malignant diseases, hypertension, diabetes, muscular dystrophy, autoimmune diseases, Alzheimer disease and so forth. In such an approach, we tried to identify the key enzyme which is essential in the disease process and to search for a specific inhibitor against the particular enzyme.[1,2]

Prolyl endopeptidase (PEP) activity in the NZB/WF1 spleen increased with progress of the disease.[3] PEP activity in the occipital lobe of Alzheimer patients was significantly higher than in normal controls, while kallikrein activity was significantly decreased.[4]

PEP was first isolated from human uterus as an entity responsible for inactivation of oxytocin and it was later reported to be distributed in a wide range of organs including brain.[5,6] Since the enzyme degrades several biologically active peptides such as vasopressin, oxytocin, thyroliberin (TRH), substance P, neurotensin, angiotensin and so on, it is considered to play an important role in the biological regulation of these peptides. After an inhibitor against the enzyme was shown to have an anti-amnesiac effect in mice, many researchers started to search for such inhibitors.[7]

In the course of screening for inhibitors of PEP, we discovered poststatin as its specific inhibitor. Poststatin was isolated from the cultured broth of *Streptomyces viridochromogenes* MH534-30F3. In this communication we report the taxonomy, production, isolation, physico-chemical properties and biological activities.

Materials and Methods

Chemicals

Chemicals employed were as follows: Diaion HP-20 from Nippon Rensui Co., Tokyo, Japan: Sephadex LH-20 from Pharmacia Fine Chemicals AB, Uppsala, Sweden: YMC-gel (ODS-A) from Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan: Glycyl-L-proline β-naphthylamide (Gly-Pro·NA) from

Bachem Feinchemikalien AG, Budendorf, Switzerland. Z-Gly-Pro·NA was synthesized from Gly-Pro·NA in our laboratory. *tert*-Butyloxycarbonyl-L-glutaminyl-L-alanyl-L-arginine 4-methylcoumaryl-7-amide (Boc-Gln-Ala-Arg·MCA), succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methylcoumaryl-7-amide (Suc-Leu-Leu-Val-Tyr·MCA) and succinyl-L-alanyl-L-prolyl-L-alanine 4-methylcoumaryl-7-amide (Suc-Ala-Pro-Ala·MCA) were obtained from Peptide Institute Inc., Minoh-shi, Japan. All other chemicals were of analytical grade.

Enzymes

PEP (EC 3.4.21.26) was prepared from porcine kidney as described by WALTER.[8] Partialy purified enzyme was used in this assay. Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), elastase (EC 3.4.21.36) and cathepsin B (EC 3.4.22.1) were obtained from Sigma Chemical Company, St. Louis, U.S.A.

Microorganism

Strain MH534-30F3 was isolated from a soil sample collected on the premises of the Institute of Microbial Chemistry, Shinagawa-ku, Tokyo and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan, under the accession No. FERM P-9446.

Taxonomic Characterization

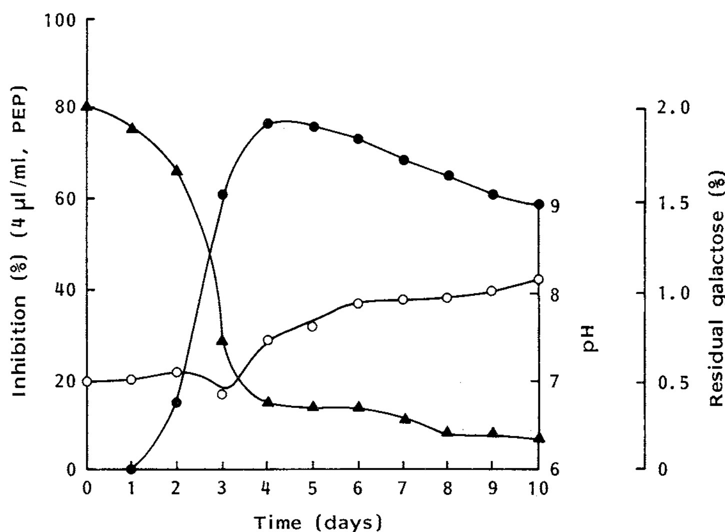
Morphological and physiological properties of the strain were examined according to SHIRLING and GOTTLIEB,[9] several other tests were also used.

Production of Poststatin

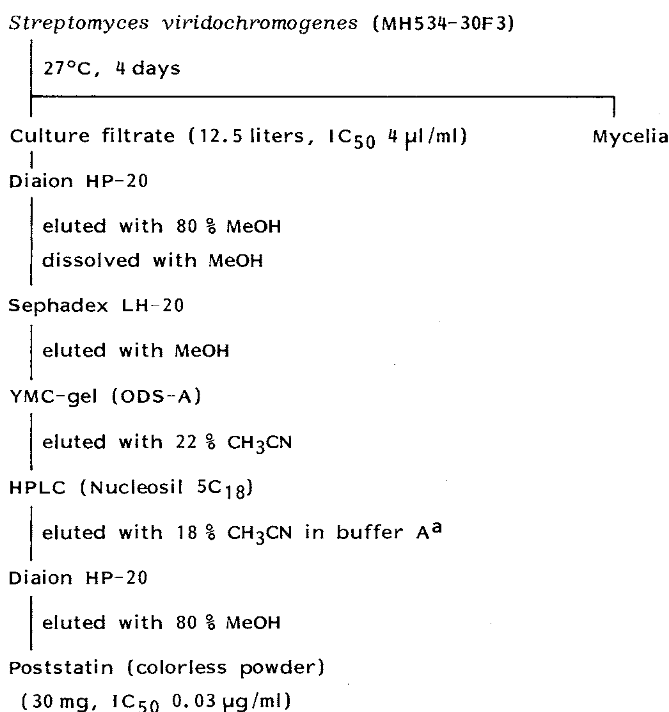
Strain MH534-30F3 was inoculated into 110 ml of a production medium consisting of Bacto Soytone 1.0%, galactose 2.0%, corn steep liquor 0.5%, dextrin 2.0%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (pH 7.4) in a 500-ml Erlenmeyer flask, and cultured at 27°C for 2 days on a rotary shaker (180 rpm). Two ml of the above seed culture was transferred to 110 ml of the same medium in a 500-ml Erlenmeyer flask and cultured for 4 days under the same conditions. The process of production of poststatin was followed by the inhibitory activity of 4 μl of broth filtrate against PEP. The time course of the production of poststatin is shown in Fig. 1.

Fig. 1. Time course of poststatin by *Streptomyces viridochromogenes* MH534-30F3.

● Inhibition, ○ pH, ▲ galactose.



Scheme 1. Isolation and purification of poststatin.



[a]Buffer A: 5 % AcOK + 1 % citric acid·H_2O (pH 5.4).

Isolation of Poststatin

The purification procedures of poststatin is shown in Scheme 1. After 4 days incubation at 27°C, the cultured broth was separated from the mycelium by centrifugation. The supernatant fraction was passed through a column of Diaion HP-20 (10% volume). The column was washed with water and eluted with 80% MeOH. The active fractions were pooled and concentrated under reduced pressure to dryness. The brownish residue was dissolved in MeOH and insoluble material was removed by centrifugation. The supernatant was subjected to a Sephadex LH-20 column and then eluted with MeOH. The active fractions were concentrated under reduced pressure. A solution of this crude powder in 22% CH_3CN was passed through a YMC-gel (ODS-A) column with the same solution. The eluate fractions containing poststatin were concentrated under reduced pressure to give a yellowish powder. This powder was dissolved in a small volume of 18% CH_3CN in buffer A (5% AcOK+1% citric acid·H_2O, pH 5.4), and the solution was subjected to reverse phase HPLC using a Nucleosil $5C_{18}$ column, and then eluted with a same buffer.

The active eluate was desalted by Diaion HP-20 adsorption followed by elution with 80% MeOH. The eluate was concentrated under a reduced pressure to give poststatin as a colorless powder.

Assay of Enzyme and Inhibitory Activity

The principle of the assay for PEP and cathepsin B activity are based on the absorbance at 525 nm of β-naphthylamine measured by microplate reader model 3550 (BIO-RAD), and for trypsin, chymotrypsin and elastase activity are based on the fluorescense (Ex 380 nm, Em 460 nm) measurement of 7-amino-4-methylcoumarin (AMC) released from the fluorogenic substrate using the Hitachi MDF-4 fluorimeter. Incubation was carried out at 37°C for 30 minutes. For the PEP and cathepsin B assay, 50 μl of 2.5 mM β-naphthylamide derivative was used as the substrate and the absorbance was determined.[6,10] For the trypsin, chymotrypsin and elastase assay, 20 μl of 2.5 mM AMC derivative was used and the fluorescense intensity was determined.[11~13]

The percent inhibition was calculated by the formula $(A-B)/A \times 100$, where A is the β-naphthylamine

and MCA liberated by the enzyme in the system without an inhibitor and B is that with an inhibitor. The IC_{50} value is the concentration of inhibitor at 50% inhibition of enzyme activity.

Inhibitors

The inhibitors used in this study were leupeptin and antipain for trypsin and cathepsin B, chymostatin for chymotrypsin and cathepsin B, and elastatinal for elastase.[1,2]

Physico-chemical Properties of Poststatin

The mp was measured by micro meltig point apparatus MP-S3 (Yanagimoto Seisakusho Co., Japan) and was uncorrected. Mass spectral data was obtained on a Hitachi M-80H mass spectrometer. The optical rotation was determined with a Perkin-Elmer 241 polarimeter using a micro-cell (light path 10 cm).

Results and Discussion

Taxonomic Characterization of the Producing Strain

Strain MH534-30F3 produces aerial mycelia forming spiral chains of spores with more than 20 spores per chain. The spores are 0.5～0.7 by 0.9～1.1 μm in size with a spiny surface. Aerial mass color of the

Table 1. Comparison of taxonomic characteristics of strain MH534-30F3 and several strains of *Streptomyces*.

	MH534-30F3	*S. viridochromogenes*	*S. chartreusis*	*S. coerulescens*
Spore chain morphology	Spirals	Spirals	Spirals	Spirals
Spore surface	Spiny	Spiny	Spiny	Spiny
Aerial mass color	White to light bluish gray	White to light bluish gray	White to light bluish gray	White to light bluish gray
Reverse color	Olive	Olive	Colorless to pale yellow	Colorless to pale yellow
Soluble pigment	None to faint brown	None to faint brown	None to faint yellow	None to faint yellow
Sensitivity of pigment	Red (HCl), faint brown (NaOH)	Red (HCl), faint brown (NaOH)	Faint yellow (HCl or NaOH)	Faint brown (HCl or NaOH)
Melanin formation				
Peptone-yeast extract-iron agar	Positive	Positive	Positive	Positive
Tryptone-yeast extract broth	Positive	Positive	Positive	Positive
Tyrosine agar	Negative	Negative	Negative	Negative
Hydrolysis of starch	Strong	Moderate	Strong	Strong
Coagulation of skim milk	Very faint	Strong	Very faint	Faint
Peptonization of skim milk	Strong	Moderate	Strong	Moderate
Liquefaction gelatin (15%)	Very faint	Very faint	Very faint	Very faint
Liquefaction of glucose-peptone-gelatin	Negative	Very faint	Negative	Negative
Nitrate reduction	Positive	Positive	Positive	Positive
Carbon utilization				
D-Glucose	+	+	+	+
L-Arabinose	+	+	+	+
D-Xylose	+	+	+	+
D-Fructose	+	+	+	+
Sucrose	+	+	+	+
Inositol	+	+	+	+
L-Rhamnose	+	+	+	+
Raffinose	+	+	+	+
D-Mannitol	+	+	+	+

+: Utilization.

colony is white to light bluish gray or light greenish gray. Growth color is colorless, pale yellow, dark yellowish brown or grayish olive on various media. The reverse color is olive. Soluble pigment was none to faint brown. The pigment was changed to red with the addition of 0.05 N HCl. Melanoid pigments were formed in peptone-yeast extract-iron agar and Tryptone-yeast extract broth, but not in tyrosine agar. The whole-cell hydrolysate of the strain showed that it contained LL-diaminopimelic acid. Based on its characteristics, strain MH534-30F3 is considered to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *S. viridochromogenes* (IMC S-0674 (ISP5110)), *Streptomyces chartreusis* (IMC S-0226 (ISP5085)) and *Streptomyces coerulescens* (IMC S-0278 (ISP5146)) are recognized to be similar to the strain MH534-30F3. Cultural and physiological characteristics and carbon utilization pattern of strain MH534-30F3 were compared with those of the three species (Table 1).

Judging from the results of reverse color and the sensitivity of pigment, the strain is most closely related to *S. viridochromogenes*[14]. However there are some differences in those cultural and physiological characreristics between the strains. These differences are not sufficient to designate the strain MH534-30F3 as a new species. Therefore, the strain is considered to be a member of *S. viridochromogenes*. Strain

Table 2. Physico-chemical properties of poststatin.

Appearance	Colorless powder
MP	169~171°C
SI-MS (*m/z*)	542 $(M+H)^+$
Molecular formula	$C_{26}H_{47}N_5O_7$
Elemental analysis ($1\frac{1}{2}$ hydrate)	
Calcd:	C 54.91, H 8.86, N 12.31
Found:	C 55.01, H 8.62, N 12.12
$[\alpha]_D^{20}$ (*c* 0.5, AcOH)	+13.9°
Rf value on TLC	
a) BuOH-AcOH-H_2O (4:1:1)	0.70
b) Acetonitrile-buffer A (7:13)	0.54
Color reaction (positive)	Ninhydrin, Rydon-Smith, 2,3,5-triphenyltetrazolium chloride, 2,4-dinitrophenylhydrazine

a): Silica gel TLC plate; Merck Art. No. 5715.
b): ODS silica gel TLC plate; Merck Art. No. 15389.

Fig. 2. Lineweaver-Burk plot of inhibition of PEP by poststatin.

● $I=4\times10^{-8}$ M, ○ $I=2\times10^{-8}$ M, ▲ I=0 M.

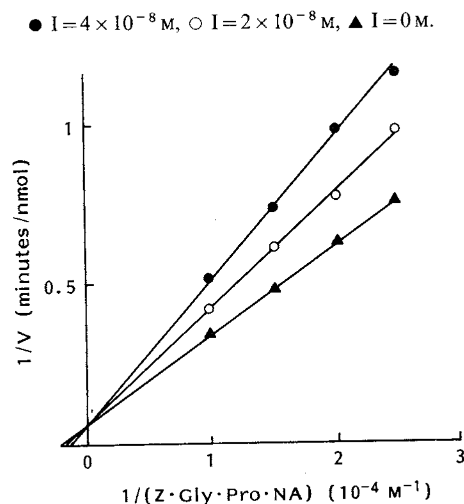


Table 3. Inhibitory activity of poststatin and other inhibitors against serine and cysteine proteinases.

Enzyme	Substrate	IC$_{50}$ (μg/ml)				
		Poststatin	Leupeptin	Antipain	Chymostatin	Elastatinal
PEP	Z-Gly-Pro·NA	0.03	>100	>100	>100	75
Trypsin	Boc-Gln-Ala-Arg·MCA	>100	1.40	0.70	>100	>100
Chymotrypsin	Suc-Leu-Leu-Val-Tyr·MCA	>100	>100	100	0.0002	>100
Elastase	Suc-Ala-Pro-Ala·MCA	>100	>100	>100	>100	0.70
Cathepsin B	Z-Arg-Arg·NA	2.10	0.04	0.15	1.40	60

Abbreviations used: Z-Gly-Pro·NA, benzyloxycarbonyl-L-proline β-naphthylamide; Boc-Gln-Ala-Arg·MCA, *tert*-butyloxycarbonyl-L-glutaminyl-L-alanyl-L-arginine 4-methylcoumaryl-7-amide; Suc-Leu-Leu-Val-Tyr·MCA, succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methylcoumaryl-7-amide; Suc-Ala-Pro-Ala·MCA, succinyl-L-alanyl-L-prolyl-L-alanine 4-methylcoumaryl-7-amide; Z-Arg-Arg·NA, benzyloxycarbonyl-L-arginy-L-arginine.

MH534-30F3 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan, under the accession No. FERM P-9446.

Production and Purification of Poststatin

The strain of *S. viridochromogenes* was cultured in Erlenmeyer flasks at 27°C for 4 days on a rotary shaker. Growth, pH and consumption of carbohydrate are plotted in a time course study of a fermentation (Fig. 1). The maximum peak of poststatin production in the flasks was obtained at 4 days, thereafter the production slowly decreased with a pH change to alkaline. The flow diagram for the isolation is shown in Scheme 1. The yield of poststatin ($C_{26}H_{47}N_5O_7$, MW 541) was 30 mg from 12.5 liters of culture filtrate.

Determination of the chemical structure of poststatin as L-valyl-L-valyl-3-amino-2-oxovaleryl-D-leucyl-L-valine are reported in a companion papers.[15~17]

Physico-chemical Properties of Poststatin

The physico-chemical properties of poststatin are summarized in Table 2. The MW and molecular formula of poststatin were determined by SI-MS and elemental analysis. Poststatin is soluble in water, methanol and dimethyl sulfoxide, but insoluble in acetone, chloroform and hexane. The spot on silica gel TLC plates is visualized by ninhydrin, Rydon-Smith, 2,3,5-triphenyltetrazolium chloride or 2,4-dinitrophenylhydrazine reagent.

Biological Activities of Poststatin

Table 3 shows the *in vitro* actions of poststatin, leupeptin, antipain, chymostatin and elastatinal. Poststatin inhibits PEP strongly and cathepsin B moderately, while leupeptin inhibits cathepsin B strongly and trypsin moderately. Antipain strongly inhibits both trypsin and cathepsin B. Chymostatin inhibits chymotrypsin strongly and cathepsin B moderately. Elastatinal inhibits elastase strongly and PEP and cathepsin B weakly. The inhibition of poststatin is competitive with substrate. The *Ki* and *Km* values for PEP are 5.6×10^{-8} M and 4×10^{-4} M, respectively (Fig. 2). Poststatin at 100 μg per ml had no antimicrobial activity. It was shown that the toxicity of this substance was low. After *iv* injection of 250 mg/kg to mice, no deaths occurred. Although it was not positively confirmed that poststatin is non-toxic, it was at least true that no pathological findings were found by autopsy. The stability of poststatin, when added to serum or other body fluids, is to be determined yet.

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