PROBESTIN, A NEW INHIBITOR OF AMINOPEPTIDASE M, PRODUCED BY *STREPTOMYCES AZUREUS* MH663-2F6

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

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Probestin has been isolated as part of a program designed to find microorganism-produced inhibitors of aminopeptidase M from *Streptomyces azureus* MH663-2F6. It was purified by use of column chromatography of Amberlite XAD-4, silica gel, YMC-gel, Toyopearl HW-40, YMC D-ODS-5 (HPLC) and then isolated as colorless powders. Probestin is competitive with the substrate, and the inhibition constant (Ki) of it was 1.9×10^{-8} M.

Since the discovery of methionine or leucine enkephalins in porcine brains, various peptides possessing opioid activities have been isolated in animals and many researchers of medical and pharmaceutical sciences are now studying intrinsic opioid peptides[1].

We also intended to elucidate the metabolism of opioid peptides and to relate it to the disease processes. Consequently we discovered that amastatin[2], bestatin[3] (Ubenimex), actinonin[4], foroxymithine[5] and so forth, inhibit enkephalin-hydrolyzing proteases[6]. In recent years, aminopeptidase M (AP-M), as an enkephalin inactivating enzyme in cerebral membranes, is the focus of interest[7]. We already reported actinonin as a specific inhibitor against AP-M. We continued screening, however, and discovered probestin as a new inhibitor[8].

As reported in this paper, we searched for new AP-M inhibitor in culture broths of microorganisms. In this communication we report the taxonomy, production, isolation, physico-chemical properties and biological activities of the inhibitor.

Materials and Methods

Chemicals

Chemicals employed were as follows: Amberlite XAD-4 from Organo Chem. Co., Tokyo, Japan; silica gel from E. Merck, Darmstadt, FRG; YMC-gel and Packed column of YMC D-ODS-5 from Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan; Toyopearl HW-40 from Tosoh MFG, Co., Ltd., Tokyo, Japan; L-leucine β-naphthylamide from Bachem Feinchemikalien AG, Budendorf, Switzerland. All other chemicals were of analytical grade.

Enzyme

AP-M (EC 3.4.11.2) of hog kidney was purchased from Boehringer Mannheim GmbH, FRG.

Microorganism

Strain MH663-2F6 was isolated from a soil sample collected in 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-9541.

Taxonomic Characterization

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

Production of Probestin

The strain MH663-2F6 was inoculated into 110 ml of a production medium consisting of potato starch 2.0%, glucose 2.0%, Soya meal 2.0%, yeast extract 0.5%, NaCl 0.25%, $CaCO_3$ 0.32%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $MgCl_2 \cdot 4H_2O$ 0.005% and $ZnCl_2 \cdot 7H_2O$ 0.005% in a 500-ml Erlenmeyer flask (pH 7.2 before sterilization), and cultured at 27°C for 2 days on a rotary shaker (180 rpm) to obtain a first seed culture. Two ml of this first seed culture were inoculated into 110 ml of the same medium in five 500-ml Erlenmeyer flasks and cultured at 27°C for 2 days to prepare a second seed culture (500 ml). Five hundred ml of the second seed culture were inoculated into the same medium (12 liters) in 30-liter jar fermenters and sterilized at 120°C for 20 minutes. The inoculated culture medium was incubated at 27°C for 48 hours under aeration (12 liters of air per minute) and with agitation (150 rpm). The process of production of probestin was followed by the inhibitory activity of 3 μl of broth filtrate against AP-M. The time course of the production of probestin is shown in Fig. 1.

Isolation of Probestin

The flow diagram for the isolation of probestin is shown in Fig. 2. The culture broth was filtered and the filtrate was adsorbed on an Amberlite XAD-4 column (10% filtrate), which was washed with water and eluted with 80% MeOH ($\times$4 column) to give active fractions. The active eluate was concentrated under a reduced pressure. The solution containing the inhibitor was adjusted to pH 2.0 with HCl, and then admixed with equal volume of BuOH, followed by agitation. The resulting extract in BuOH was concentrated under a reduced pressure to obtain crude brownish powder. The crude powder was suspended in a solvent mixture of BuOAc - BuOH - AcOH - H_2O (2 : 4 : 1 : 1). The suspension was passed through a column of silica gel ($\times$50 w/w powder) which had been packed with the same solvent mixture. The eluate was concentrated under a reduced pressure to give pale brownish powder containing probestin. The crude powder was dissolved in H_2O, and the solution was subjected to a reversed phase column chromatography on a column of YMC-gel (2.3 $\times$ 17 cm) which had been packed with H_2O. Active fractions were eluted gradiently with 0~50% MeOH. The eluate fractions containing probestin were concentrated under a reduced pressure to give yellowish powder. This powder was dissolved in a small volume of MeOH, and the solution was subjected to a Toyopearl HW-40 column (3.3 $\times$ 58 cm) chromatography developed with

Fig. 1. Time course of probestin production by *Streptomyces azureus* MH663-2F6.

● Inhibition, ○ pH, ▲ glucose, △ starch.

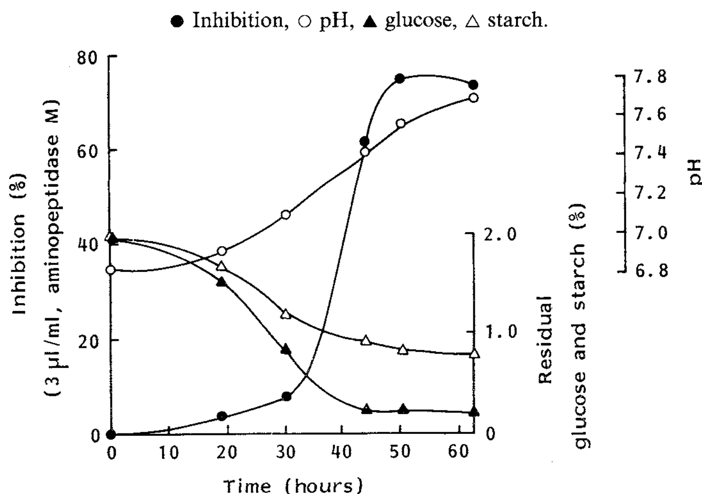
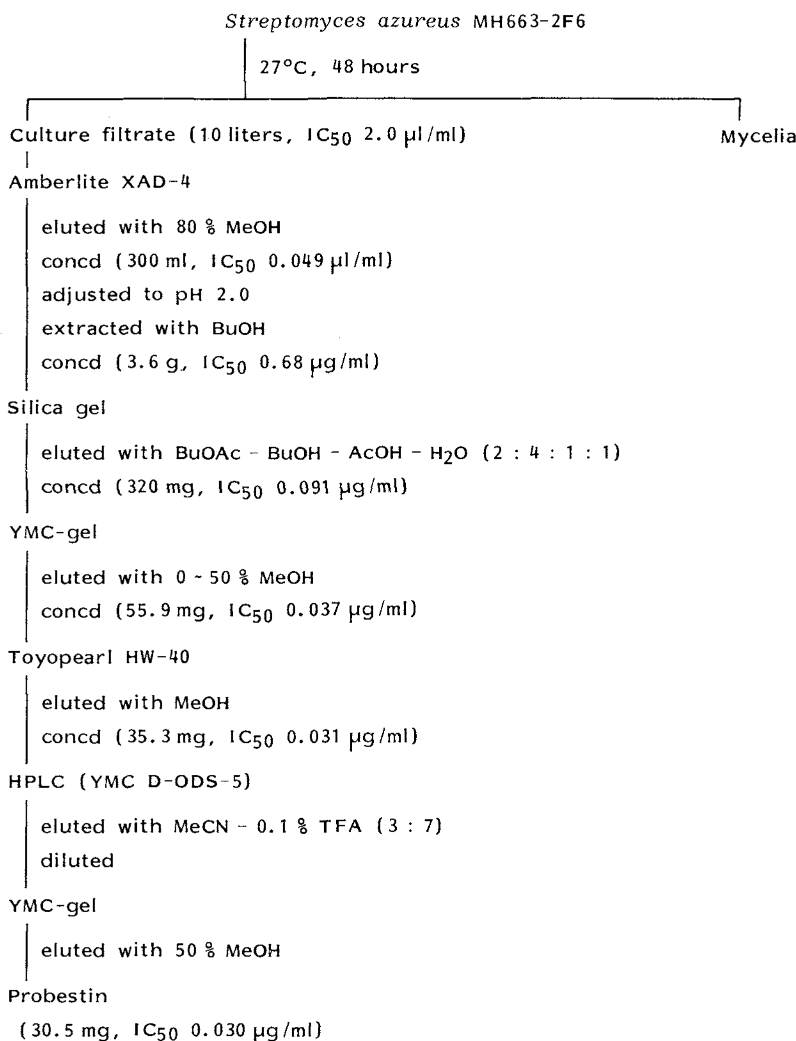


Fig. 2. Isolation of probestin.



MeOH to afford colorless powder. The powder was further purified by a reversed phase HPLC using a YMC D-ODS-5 column (2 × 25 cm, flow rate 6.3 ml/minute, using Waters ALC/GPC 200 system) with a 3:7 mixture of MeCN and 0.1% TFA. The active eluate was passed through a reversed phase column chromatography on a column of YMC-gel (2.3 × 17 cm) which had been packed with H_2O. This was followed by successive elution with 50% MeOH to collect the fractions containing probestin. The eluate was concentrated under a reduced pressure to give probestin as colorless powder.

Assay for AP-M and Inhibitory Activity

AP-M activity was measured as reported previously[4]. The reaction mixture (total 1 ml) contained 0.25 ml of 2 mM L-leucine β-naphthylamide, 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.0) and 0.2 ml of water or aqueous solution containing the test compound. The mixture was incubated at 37°C for 3 minutes, and 0.05 ml of AP-M solution (1 mU) was added. After 30 minutes, the enzymatic reaction was terminated by the addition of 1 ml of 1 M acetate buffered solution (pH 4.2) containing 10% of Tween 20 and 0.1% of Fast garnet GBC (*o*-aminoazotoluene, diazonium salt). After allowing the mixture to stand at room temperature for 15 minutes, the absorbance of light at 525 nm is measured.

AP-A, B and leucine aminopeptidase (Leu-AP) activities were measured as reported previously[2,3].

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

by the enzymatic reaction 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.

Physico-chemical Properties of Probestin

The mp was measured by micro melting point apparatus MP-S3 (Yanagimoto Seisakusyo Co., Japan) and was uncorrected. Mass spectrum was carried out 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 MH663-2F6 has branched substrate mycelia, from which aerial hyphae develop in the form of open spirals. No whirl-formation was observed. Matured spore-chains usually bear more than 20 conical spores. Spores ranged about 0.7~0.8 by 0.7~0.9 μm in size and have smooth surface. Aerial mass color of the colony was white to bright bluish gray. The color of vegetative growth was pale yellow to pale yellowish brown or pale brown. Soluble pigments were none to faintly tinged with brown. Melanoid pigments were observed on Tryptone-yeast extract broth (ISP-medium 1) and on peptone-yeast extract-iron agar (ISP-medium 6), but not on tyrosine agar (ISP-medium 7).

Table 1. Comparison of taxonomic characteristics of strain MH663-2F6 with *Streptomyces azureus* and *Streptomyces caelestis*.

	MH663-2F6	*S. azureus* IMC S-0225 (ISP 5084)	*S. caelestis* IMC S-0208 (ISP 5106)
Spore chain morphology	Spirals	Spirals	Spirals
Spore surface	Smooth	Smooth	Smooth
Aerial mass color	White to bright gray	White to grayish bluish green	White to bright gray
Color of vegetative growth	Pale yellow to pale yellowish brown or pale brown	Pale yellowish brown to pale brown	Pale yellowish brown to pale brown
Soluble pigment	None to faintly tinged with brown	None to faintly tinged with brown	None to faintly tinged with brown
Melanin formation:			
ISP-medium 1	Positive	Positive	Positive
ISP-medium 6	Positive	Positive	Positive
ISP-medium 7	Negative	Negative	Negative
Hydrolysis of starch	Strong	Strong	Strong
Coagulation of skim milk	Negative	Negative	Negative
Peptonization of skim milk	Faint	Faint	Faint
Liquefaction of gelatin:			
Plain gelatin	Negative	Negative	Negative
Glucose-peptone-gelatin	Positive	Positive	Positive
Nitrate reduction	Positive	Positive	Positive
Carbon utilization:			
D-Glucose	+	+	+
L-Arabinose	+	+	+
D-Xylose	+	+	+
D-Fructose	+	+	+
D-Mannitol	+	+	+
Inositol	+	+	+
L-Rhamnose	+	+	+
Raffinose	+	+	+
Sucrose	+	+	+

+: Utilized.

The whole-cell hydrolysate of the strain showed that it contained LL-diaminopimelic acid. Based on its characteristics, strain MH663-2F6 is considered to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *Streptomyces azureus* and *Streptomyces caelestis* are recognized to be similar to the strain MH663-2F6. The results of comparison of the strain MH663-2F6 and these two species are summarized in Table 1. As will be apparent from Table 1, the strain MH663-2F6 is closely related to *S. azureus* and *S. caelestis* and in their microbiological properties. The strain MH663-2F6 is different from *S. caelestis* in the utilization of D-mannitol, and it is different from *S. azureus* with respect to the color characteristics of aerial hyphae. The development of open spirals in the aerial hyphae, which is one of the morphological characteristics of the strain MH663-2F6, is also equally observed with *S. azureus*. It appears under microscopic observation that the strain MH663-2F6 and *S. caelestis* produce poorly open spirals, with the open spirals having 1~2 turns and with the spore chains being short. Therefore, we estimated that the strain MH663-2F6 belonged to *S. azureus*, and it was designated as *S. azureus* MH663-2F6.

Production and Isolation of Probestin

The strain of *S. azureus* MH663-2F6 was cultured in a jar fermenter at 27°C for 48 hours. The time course of the production is shown in Fig. 1. The maximum peak of probestin production in the jar fermenter was obtained at 48 hours and thereafter the production slowly decreased with a pH change to alkaline. The flow diagram for the isolation is shown in Fig. 2. The yield of pure probestin was 28.8 mg. The purity of each preparation was confirmed by TLC and HPLC.

Physico-chemical Properties of Probestin

The physico-chemical properties of probestin are summarized in Table 2. The MW and formula were determined to be $C_{26}H_{38}N_4O_6$ (MW 502.61) by SI-MS, elemental analysis and ^{13}C NMR study.

Table 2. Physico-chemical properties of probestin.

Appearance	Colorless powder
MP	168~170°C
SI-MS (*m/z*)	503 (M+H)
MW	502.61
Molecular formula	$C_{26}H_{38}N_4O_6$
Elemental analysis	
Calcd for	
$C_{26}H_{38}N_4O_6 \cdot H_2O$:	C 59.98, H 7.74, N 10.76
Found:	C 60.25, H 7.75, N 10.97
$[\alpha]_D^{25}$ (*c* 1.0, MeOH)	−117.0°
Color reaction	Ninhydrin, Greig-Reaback
Rf value	
I	0.39
II	0.57
Rm[a] value (alanine=1.0)	1.0

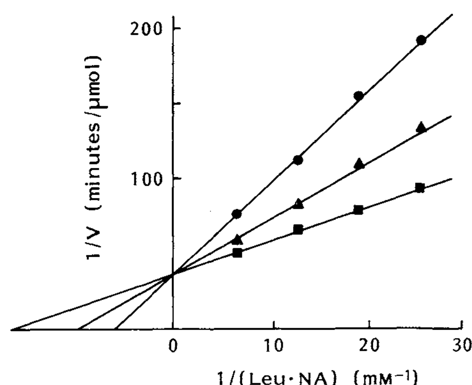
I: On silica gel TLC plate (Merck Art. No. 5715) with BuOH - AcOH - H_2O (4:1:1) as eluent.
II: On silica gel TLC plate (Merck Art. No. 15389) with CH_3CN - buffered solution of 5% CH_3COOK and 1% citric acid monohydrate (3:5) as eluent.
[a] HVPE in HCOOH - CH_3COOH - H_2O (1:3:36) under 800V for 15 minutes.

Table 3. Inhibitory activities of probestin, actinonin, amastatin and bestatin against aminopeptidases.

	IC$_{50}$ (μg/ml)			
	AP-M	Leu-AP	AP-A	AP-B
Probestin	0.030	0.090	>100	37
Actinonin	0.40	1.0	>100	>100
Amastatin	0.58	0.50	0.54	>100
Bestatin	6.2	0.010	>100	0.050

Fig. 3. Lineweaver-Burk plot of inhibition of AP-M by probestin.

● I=0.01 μg/ml, ▲ I=0.005 μg/ml, ■ I=0 μg/ml.



Probestin is soluble in water methanol and dimethyl sulfoxide, but insoluble in acetone, ethyl acetate, chloroform and hexane.

Determination of the structure of probestin will be described in the following paper[10].

Biological Activities of Probestin

The inhibitory activities of probestin and various inhibitors of aminopeptidases are shown in Table 3. It inhibits not only AP-M but also Leu-AP and AP-B. As shown in Fig. 3. It is competitive with substrate. The *Ki* value of probestin is 1.9×10^{-8} M. It had no antimicrobial activity at 100 μg/ml. It has low toxicity; there were no deaths after iv injection of mice with 250 mg/kg.

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