LEUHISTIN, A NEW INHIBITOR OF AMINOPEPTIDASE M, PRODUCED BY *Bacillus laterosporus* BMI156-14F1

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

TAKAAKI AOYAGI, SHIGEMI YOSHIDA*, NAOKO MATSUDA, TAKAKO IKEDA, MASA HAMADA and TOMIO TAKEUCHI

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

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Leuhistin has been isolated from the culture broth of *Bacillus laterosporus* BMI156-14F1 as part of a program designed to find microorganism-produced inhibitors of aminopeptidase M (AP-M). It was purified by use of column chromatography on Sepabeads SP206, Amberlite IRC-50, MCI gel CHP-20P and Sephadex G-10 and then isolated as colorless needles. Leuhistin inhibits AP-M strongly and it also inhibits AP-A and AP-B weakly. It is competitive with the substrate, and the inhibition constant (*Ki*) was 2.3×10^{-7} M.

For many years, the authors have screened for inhibitors against various proteases including ectopeptidases and have found various kinds of inhibitors. Inhibitors of the ectoenzyme were found which modify cellular functions including immune reactions. The findings provide a new approach to the studies of regulatory mechanisms of the cells. Thus it is expected that the studies on enzyme inhibitors will contribute greatly to the progress of biological science[1].

In recent years, aminopeptidase M (AP-M), an inactivating enzyme of bioactive peptides in cerebral membranes, has been the focus of interest[2]. Actinonin[3] and probestin[4,5] were reported as specific inhibitors against AP-M and more recently leuhistin[6], a new inhibitor, was discovered.

In this communication, the taxonomy, production, isolation, physico-chemical properties and biological activities of the inhibitor are reported.

Materials and Methods

Chemicals

Chemicals employed were as follows: Sepabeads SP206 and MCI gel CHP-20P from Nippon Rensui Co., Tokyo, Japan; Amberlite IRC-50 from Organo Co., Tokyo, Japan; Sephadex G-10 from Pharmacia Fine Chemicals Co., Tokyo, Japan. All other chemicals were of analytical grade.

Emzyme

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

Microorganism

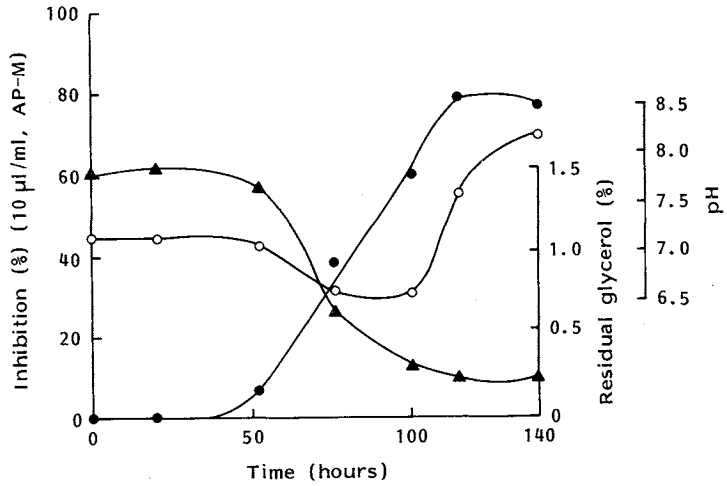
Strain BMI156-14F1 was isolated from a soil sample collected in Bunkyo-ku, Tokyo and has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba-shi, Japan, under the accession No. FERM P-10193.

Taxonomic Characterization

Taxonomic studies of the strain were carried out according to the methods described in BERGEY'S

Fig. 1. Time course of leuhistin production by *Bacillus laterosporus* BMI156-14F1.

● Inhibition, ○ pH, ▲ glycerol.



Manual of Determinative Bacteriology 8th Ed.[7], Manual for Identification of Medical Bacteria 2nd Ed.[8], and Taxonomy and Identification of Microorganisms[9], several other tests were also used.

Production of Leuhistin

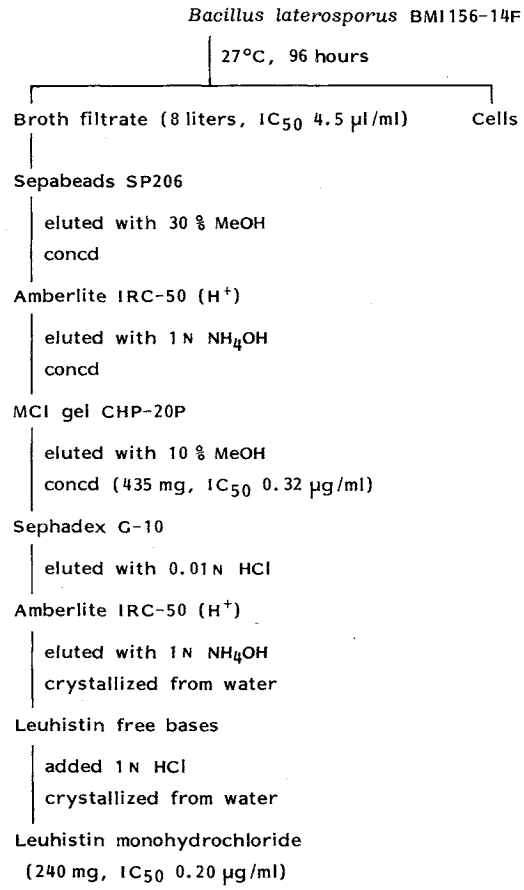
The strain BMI156-14F1 was inoculated into 110 ml of a seed medium consisting of glycerol 1.5%, Pharmamedia 1.0%, dry yeast 1.2% and $CaCO_3$ 0.2% (pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask, and cultured at 27°C for 2 days on a rotary shaker (180 rpm) to obtain a seed culture. Two ml of this seed culture were inoculated into 110 ml of the production medium consisting of glycerol 1.5%, Pharmamedia 1.0%, dry yeast 1.2%, L-leucine 0.5%, L-histidine·HCl 0.2% and $CaCO_3$ 0.2% (pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask and cultured for 115 hours under the same condition.

The process of production of leuhistin was followed by the inhibitory activity of 10 µl of broth filtrate against AP-M. The time course of the production of leuhistin is shown in Fig. 1.

Isolation of Leuhistin

The flow diagram for the isolation of leuhistin is shown in Fig. 2. The culture broth was filtered and the filtrate was adsorbed on a Sepabeads SP206 column (10% filtrate), which was washed with water and eluted with 30% MeOH (×3 column) to give active fractions. The active eluate was concentrated under reduced pressure to remove MeOH. The resulting solution was adsorbed on an Amberlite

Fig. 2. Isolation of leuhistin.



IRC-50 (free acid form) column (4.5 × 31 cm), which was washed with water and eluted with 1 N ammonium hydroxide. The eluate was concentrated under reduced pressure to give a brownish powder containing leuhistin. The powder was dissolved in a small volume of 10% MeOH, and the solution was applied to a column of MCI gel CHP-20P (3.9 × 84 cm), eluted with 10% MeOH and concentrated to give pale brownish powder. This powder was dissolved in a small volume of 0.01 N HCl, and the solution was applied to a Sephadex G-10 column (3.9 × 84 cm) and eluted with 0.01 N HCl. The active eluate was desalted on an Amberlite IRC-50 (free acid from) column (3.9 × 17 cm) followed by elution with 1 N ammonium hydroxide. The effluent was concentrated under reduced pressure to a small volume and kept in a refrigerator to give colorless needles of leuhistin free base.

Preparation of Leuhistin Monohydrochloride

A solution of HCl (1 N, 0.5 ml) was added to a solution of 120.5 mg of leuhistin free base in 12 ml of water. The solution (pH 3.1) was lyophilized to give colorless powder of leuhistin monohydrochloride. A solution of this powder in a small volume of water was kept in a refrigerator to give colorless needles of leuhistin monohydrochloride.

Assay for AP-M and Inhibitory Activity

Inhibitory activities against AP-M, AP-A, AP-B and Leu-AP were measured as reported previously[4].

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 the value obtained with an inhibitor. The IC_{50} value is the concentration of inhibitor at 50% inhibition of enzyme activity.

Physico-chemical Properties of Leuhistin

The mp was measured by micro melting point apparatus MP-S3 (Yanagimoto Seisakusho Co., Japan) and was uncorrected. MS 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 BMI156-14F1 showed the following morphological, cultural and physiological properties.

Morphological Characteristics: Cultivation were performed at 30°C with the exception of gelatin stab culture (20°C). Nutrient agar colony; slightly glistening, opaque, circular with irregular circumference, pale yellow to pale brown, rugose surface after 3 days incubation, no diffusible pigments. Nutrient gelatin stab; stratiform liquefaction. Milk; coagulation at a week incubation, peptonization with pellicle growth.

Physiological Characteristics: Nitrate reduction, casein hydrolysis, and oxidase and catalase formation were positive. Denitrification, methyl red test (glucose-peptone broth), acetylmethylcarbinol formation (glucose-peptone broth), H_2S formation, starch hydrolysis, citrate utilization (KOSER's and CHRISTENSEN's media), urease formation, and lysozyme sensitivity (peptone 1%, meat extract 1%, NaCl 0.5% and lysozyme 0.001%, pH 7.0) were negative. Indole formation was doubtful. Ammonium sulfate but not sodium nitrate were utilized as sole nitrogen source. Facultatively anaerobic. HUGH-LEIFSON's OF test was of the fermentative type. Acid but no gas (sugar 1%, yeast extract 0.02%, inorganic salts 0.14% and agar 2% with bromocresol purple as pH indicator) was produced from D-glucose, D-mannose, D-fructose, D-mannitol, maltose, trehalose and glycerol. Neither acid nor gas was produced from L-arabinose, D-xylose, D-galactose, D-sorbitol, sucrose, lactose, *myo*-inositol or starch. Pale yellowish fluorescent pigments were produced on Kings A and B media. The temperature range for growth was between 20°C and 45°C with optimum growth at around 35°C. The pH range for growth was between 5.7 and 9.0 with optimum growth at around pH 7.0.

Table 1. Comparison of characteristics of strain BMI156-14F1 with *Bacillus laterosporus* and *B. brevis*.

Category	BMI156-14F1	*B. laterosporus*	*B. brevis*
Form of spore	Ellipsoidal	Ellipsoidal or cylindrical	Ellipsoidal or cylindrical
Swelling of cell at spore formation	+	+	+
Position of spore	Central	Central	Central, subterminal or terminal
Acid formation from glucose	+	+	+
Gas formation from glucose	−	−	−
Acetoin formation from glucose	−	−	−
Cell size (μm)	0.6~1.0×2.0~4.5	0.5~0.8×2.0~5.0	0.6~0.9×1.5~4.0
Motility	+	+	+
Growth temperature (°C)	20~45	(15~20)~(35~50)	(10~35)~(40~60)
Acid formation from L-arabinose and D-xylose	−	−	−
Acid formation from D-mannitol	+	+	d
Anaerobic growth	+	+	−
Reduction of nitrate	+	+	d
Gram-stain	Inconstant	Inconstant	Inconstant
Hydrolysis of starch	−	−	−
Formation of indole	−	d	−
Utilization of citric acid	−	−	+

+: Positive in 90 to 100% of sample, −: negative in 90 to 100% of sample, d: positive in 11 to 89% of sample.

The above characteristics indicated that this strain was resulted to *Bacillus laterosporus* or *Bacillus brevis*. The comparison of the characteristics of the strain BMI156-14F1 with those of *B. laterosporus* and *B. brevis* is shown in Table 1. Strain BMI156-14F1 is more closely related to *B. laterosporus* than *B. brevis* in its possession of a parasporal body, growth under unaerobic conditions and negative utilization of citric acid. Therefore, strain BMI156-14FI should be classified as a strain of *B. laterosporus*.

Table 2. Physico-chemical properties of leuhistin monohydrochloride.

Appearance	Colorless needles
MP	180~183°C
SI-MS (*m/z*)	242 $(M+H)^+$
Molecular formula[a]	$C_{11}H_{19}N_3O_3$
Elemental analysis[a]	
Calcd for $C_{11}H_{19}N_3O_3$:	C 54.76, H 7.94, N 17.41
Found:	C 54.71, H 8.09, N 17.25
$[\alpha]_D^{25}$ (*c* 1.0, MeOH)	−51.4°
UV spectrum	End absorption
Color reaction (positive)	Ninhydrin, Pauly
Rf[b] value	0.39
Rm[c] value (alanine = 1.0, pH 1.8)	1.3
Solubility	
Soluble:	H_2O, MeOH, EtOH, DMSO
Insoluble:	Me_2CO, EtOAc, $CHCl_3$, hexane

[a] Data for leuhistin free base.
[b] On silica gel TLC (BuOH - AcOH - H_2O, 2 : 1 : 1).
[c] HVPE.

Production and Isolation of Leuhistin

The strain of *B. laterosporus* BMI156-14F1 was cultured in an Erlenmeyer flask at 27°C for 115 hours on a rotary shaker. The time cource of the production of leuhistin is shown in Fig. 1. The maximum peak of leuhistin production in Erlenmeyer flask was obtained at 115 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 leuhistin monohydrochloride was 240 mg. The purity of each preparation was confirmed by the inhibitory activity against AP-M.

Physico-chemical Properties of Leuhistin Monohydrochloride

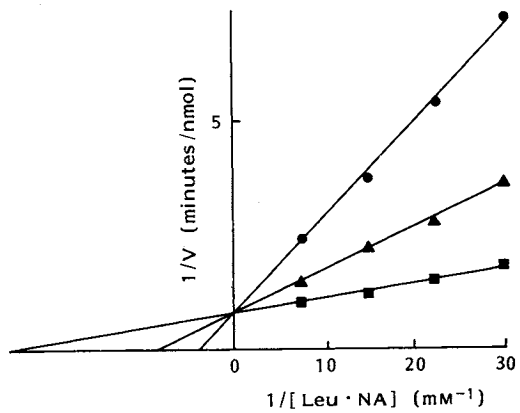
The physico-chemical properties of leuhistin are summarized in Table 2. The MW and formula were

Table 3. Inhibitory activities of various inhibitors.

	IC_{50} (μg/ml)			
	AP-M	Leu-AP	AP-A	AP-B
Leuhistin	0.20	>100	10	13
Probestin	0.030	0.09	>100	37
Prostatin	0.028	0.30	90	60
Actinonin	0.40	1.0	>100	>100
Amastatin	0.58	0.5	0.54	>100
Ubenimex	6.2	0.01	>100	0.05

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

● I=0.2 μg/ml, ▲ I=0.05 μg/ml, ■ I=0 μg/ml.



determined to be $C_{11}H_{19}N_3O_3$ (MW 241.29) by SI-MS and elemental analysis. Leuhistin monohydrochloride is soluble in water, methanol, ethanol and dimethyl sulfoxide, but insoluble in acetone, ethyl acetate, chlorofolm and hexane.

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

Biological Activity of Leuhistin

The inhibitory activities of leuhistin and various inhibitors of aminopeptidases are shown in Table 3. It inhibited AP-M weakly in comparison with probestin, but did not inhibit Leu-AP at 100 μg/ml. Leuhistin is a specific inhibitor of AP-M, and was not inhibitory to Leu-AP at concentrations of more than 1,000-fold that of the IC_{50} value against AP-M. As shown in Fig. 3, it is competitive with substrate, the *Ki* value of leuhistin being 2.3×10^{-7} M. Leuhistin had no antimicrobial activity at 100 μg/ml and was well tolerated by mice on intraperitoneal injection of 250 mg/kg.

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