NAGSTATIN, A NEW INHIBITOR OF *N*-ACETYL-β-D-GLUCOSAMINIDASE, PRODUCED BY *Streptomyces amakusaensis* MG846-fF3

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

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Nagstatin, a new inhibitor of *N*-acetyl-β-D-glucosaminidase (NAG-ase) was discovered in the fermentation broth of *Streptomyces amakusaensis* MG846-fF3. It was purified by chromatography on Dowex 50W, Avicel and Sephadex LH-20 followed by the treatment of active carbon and then isolated as colorless powder. Nagstatin has the molecular formula of $C_{12}H_{17}N_3O_6$. It is competitive with the substrate, and the inhibition constant (*Ki*) was 1.7×10^{-8} M.

In several diseases such as diabetes mellitus[1], leukemia[2] and cancer[3] *N*-acetyl-β-D-glucosaminidase (NAG-ase, EC 3.2.1.30) activity in serum has been reported to increase. In addition, such increases are useful indicators for hepatic disease and pregnancy[4~6]. NAG-ase, an exoglycosidase, is located in lysozome and releases NAG from glycoproteins and glycolipids. In recent years many isozymes have been discovered[7]. For elucidating the cause and process of the above diseases, it seems important to examine behavior of these isozymes. If we succeed in finding out specific inhibitors against these NAG-ase, these inhibitors may enable us to understand the mechanisms or the processes of the intractable diseases such as nephritis, cancer, immune disorder and so forth and may also provide us therapeutic approaches to them.

In the couse of screening for inhibitors of NAG-ase, we discovered nagstatin as a specific inhibitor. 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: Active carbon from Wako Pure Chemical Industries, Ltd., Osaka, Japan; Dowex 50W from Muromachi Kagaku Kogyo Kaisha, Ltd., Tokyo, Japan; Avicel from Funakoshi Pharmaceutical Co., Tokyo, Japan; Sephadex LH-20 from Pharmacia Fine Chemicals AB, Uppsala, Sweden; TLC-plate Silica gel F_{254} (0.25 mm thickness) from E. Merck, Darmstadt, FRG; *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (NP-GlcNAc) from Sigma Chemical Co., St. Louis, U.S.A.

Enzymes

NAG-ase was prepared from hog kidney as described by TARENTINO *et al.*[8]. Partially purified enzyme was used in this assay.

Microorganism

Strain MG846-fF3 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, Japan, under the accession No. FERM P-8095.

Taxonomic Characterization

Morphological and physiological properties of the strain were examined according to SHIRLING and GOTTLIEB[9].

Production of Nagstatin

A loopful of spores of the strain MG846-fF3 was inoculated into 110 ml of a production medium consisting of galactose 2.0%, dextrin (Wako Pure Chemical Industries, Ltd., Osaka, Japan, practical grade) 2.0%, Bacto Soytone 1.0%, corn steep liquor (Iwaki Co., Ltd., Tokyo, Japan) 0.5%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (pH 7.4) in a 500-ml baffled Erlenmeyer flask, and incubated at 27°C for 2 days on a rotary shaker (180 rpm). One ml of this seed culture was transferred to 110 ml of the same medium in a 500-ml baffled Erlenmyer flask and cultured for 4 days under same conditions.

Isolation of Nagstatin

The culture broth was filtered through Celite and the culture filtrate was adsorbed on active carbon (290 g) and eluted with MeOH (pH 2). The eluate was concentrated to dryness under reduced pressure. The dried material was chromatographed on Dowex 50W (3×43 cm) with a linear gradient of 0.02 M pyridine-acetic acid (pH 3.10) to 0.5 M pyridine-acetic acid (pH 4.72). The eluate was concentrated to dryness under reduced pressure and further purified by a column of Avicel (3×43 cm) with BuOAc-BuOH-AcOH-H_2O (3:4:1:1). The eluate fractions containing nagstatin were evaporated to give pale brownish powder. This powder was dissolved in a small volume of 80% aq MeOH, and the solution was subjected to Sephadex LH-20 column (1.6×150 cm) chromatography developed with same solvent. The eluate was concentrated and lyophilized to give nagstatin as colorless powder.

Assay for *N*-Acetyl-β-D-glucosaminidase (NAG-ase) and Inhibitory Activity

The activity of NAG-ase was determined colorimetrically by measuring the amount of *p*-nitrophenol that has been liberated when NP-GlcNAc was used as a substrate[8]. The reaction mixture (total 1.0 ml) contained 0.5 ml of 0.1 M sodium citrate buffer (pH 4.5), 0.05 ml of 25 mM NP-GlcNAc and water or aqueous solution containing the test compound. The mixture was incubated at 37°C for 3 minutes, and 0.01 ml of NAG-ase was added. After 30 minutes of reaction, 1.0 ml of 0.4 M glycine-sodium hydroxide buffer (pH 10.5) was added and the absorbance of the liberated nitrophenol measured at 400 nm.

The percent inhibition was calculated by the formula $(A-B)/A \times 100$, where A is the nitrophenol liberated by the enzyme without an inhibitor and B is that with an inhibitor. The IC_{50} value is the concentration of inhibitor at 50% of enzyme activity.

Physico-chemical Properties of Nagstatin

Melting point was taken using a Yanaco MP-S3 apparatus (Yanagimoto Seisakusho Co., Japan) and was uncorrected. UV spectrum was recorded on a Beckman DU-8 spectrophotometer, and IR spectrum on a Hitachi 260-10 spectrophotometer. Optical rotation was measured on a Perkin-Elmer 241 polarimeter using a micro-cell (light path 10 cm). Mass spectrum was obtained on a Hitachi M-80H mass spectrometer.

Results and Discussion

Taxonomic Characterization of the Producing Strain

Strain MG846-fF3 has branched substrate mycelia, from which aerial hyphae develop in the form of hooks or open spirals. No whorl-formation was observed. Matured spore-chains usually bear more than 20 conical spores. Spores ranged from about 0.5~0.6 by 0.7~0.8 μm in size and have smooth surface.

Table 1. Comparison of taxonomic characteristics of strain MG846-fF3 with *Streptomyces amakusaensis*.

	MG846-fF3	*S. amakusaensis* IMC S-0077 (ISP 5219)
Spore chain morphology	Hooks-spirals	Hooks-spirals
Spore surface	Smooth	Smooth
Aerial mass color	White to bright olive	White to bluish white
Color of vegetative growth	Colorless to light brown	Colorless to light brown
Soluble pigment	Colorless to faint yellow	Colorless to faint yellow
Melanin formation:		
ISP-medium 1	Negative	Negative
ISP-medium 6	Negative	Negative
ISP-medium 7	Negative	Negative
Hydrolysis of starch	Positive	Positive
Coagulation of skim milk	Positive	Positive
Peptonization of skim milk	Positive	Positive
Liquefaction of gelatin:		
Plain gelatin	Positive	Positive
Glucose-petone-gelatin	Negative	Negative
Nitrate reduction	Negative	Negative
Carbon utilization:		
D-Glucose	+	+
L-Arabinose	−	−
D-Xylose	+	−
D-Fructose	−	−
Sucrose	−	−
Inositol	+	−
L-Rhamnose	−	−
Raffinose	−	−
D-Mannitol	−	−

+, Utilization; −, no utilization.

Aerial mass color of the colony was white to bright olive gray and the color of vegetative growth was colorless to light brown. Soluble pigments were none to faint yellow. Melanoid pigments were not formed. The protein-decomposing action was of a middle to high strength, and the starch hydrolysis was of a high degree. The whole-cell hydrolysate of the strain showed that it contained LL-diaminopimelic acid.

Based on its characteristic, strain MG846-fF3 is considered to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *Streptomyces amakusaensis* is recognized to be similar to the strain MG846-fF3. The results of comparison of the strain MG846-fF3 and *S. amakusaensis* IMC S-0077 (ISP 5219) are summarized in Table 1. As will be apparent from Table 1, the strain MG846-fF3 is colsely related to *S. amakusaensis* in its microbiological properties except for the utilizations of D-xylose and inositol. These differences are not sufficient to designate the strain MG846-fF3

Table 2. Physico-chemical properties of nagstatin.

Appearance	Colorless powder
MP	190～195°C
$[\alpha]_D^{25}$	+46.2° (*c* 0.5, H_2O)
SI-MS (*m/z*)	322 $(M+Na)^+$
Elemental analysis	
Found:	C 48.58, H 5.80, N 13.96, O 31.99
Calcd for $C_{12}H_{17}N_3O_6$:	C 48.16, H 5.73, N 14.04, O 32.08
UV λ_{max}^{MeOH} nm (log ε)	225 (3.52)
IR ν_{max}^{KBr} cm^{-1}	3412, 2928, 1670, 1590, 1376, 1122
Rf value[a]	0.45
Rm value (alanine = 1.0)[b]	0.79
Color reaction	Greig-Leaback
Solubility	Soluble: H_2O, DMSO, MeOH Insoluble: $CHCl_3$

[a] Silica gel TLC: 1-PrOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 6).

[b] HVPE: HCOOH - CH_3COOH - H_2O (1 : 3 : 36, pH 1.6), 800 V, 15 minutes.

as a new species. Therefore, we estimated that the strain MG846-fF3 belong to *S. amakusaensis*, and it was designated as *S. amakusaensis* MG846-fF3.

Production and Isolation of Nagstatin

The strain *S. amakusaensis* MG846-fF3 was cultured in baffled Erlenmeyer flasks at 27°C for 4 days on a rotary shaker. The maximum peak of nagstatin production in the flasks was obtained at 4 days. From the culture filtrate (14.5 liters), nagstatin was isolated as shown in Fig. 1. The total yield of nagstatin was 207 mg. The purity of each preparation was confirmed by TLC developed with 1-PrOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 6). The Rf value of nagstatin is 0.45.

Physico-chemical Properties of Nagstatin

The physico-chemical properties of nagstatin are summarized in Table 2. The molecular weight and formula were determined to be $C_{12}H_{17}N_3O_6$ (MW 299.3) by SI-MS and elemental analysis. Nagstatin is highly soluble in water, and sparingly soluble in methanol and dimethyl sulfoxide, and insoluble in chloroform.

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

Fig. 1. Isolation of nagstatin.

S. amakusaensis MG846-fF3
↓ 27°C, 96 hours
Culture filtrate (14.5 liters, IC_{50} = 0.02 μl/ml) Mycelia
↓ Active carbon treatment
↓ MeOH (pH 2)
Dowex 50W chromatography
↓ Pyridine - AcOH linear gradient 0.02 M (pH 3.10) to 0.5 M (pH 4.72)
Avicel chromatography
↓ BuOAc - BuOH - AcOH - H_2O (3 : 4 : 1 : 1)
Sephadex LH-20
↓ 80% aq MeOH
Nagstatin (207 mg, IC_{50} = 0.0012 μg/ml)

Fig. 2. Lineweaver-Burk plot of inhibition of NAG-ase by nagstatin.

□ I = 2.4×10^{-8} M, ● I = 1.2×10^{-8} M, ○ I = 0 M.

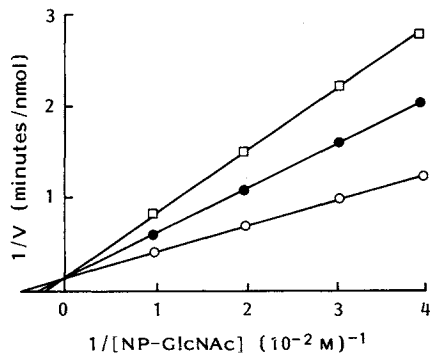

Table 3. Inhibitory activity of various inhibitors against glycosidases.

Inhibitor	IC_{50} (μg/ml)			
	Sialidase[a]	β-D-Galactosidase[b]	α-D-Mannosidase[c]	*N*-acetyl-β-D-Glucosaminidase[d]
Siastatin B	3.0	>100	>100	>100
Pyridindolol	>100	1.8	>100	>100
Mannostatin A	>100	>100	0.02	>100
Mannostatin B	>100	>100	0.02	>100
Nagstatin	>100	>100	>100	0.0012

[a] *Clostridium perfringens*.
[b] Bovine liver.
[c] Rat epididymis.
[d] Hog kidney.

Biological Activities of Nagstatin

As shown in Fig. 2, the inhibition of nagstatin against NAG-ase is competitive with substrate. The *Ki* value of nagstatin is 1.7×10^{-8} M. The spectrum of various glycosidase inhibitors which were found in microbial culture fluids is shown in Table 3. Siastatin B[11] is a specific inhibitor against sialidase, pyridindolol[12] against β-D-galactosidase, mannostatins A and B[13] against α-D-mannosidase and nagstatin against *N*-acetyl-β-D-glucosaminidase, resepctively.

Nagstatin had no significant antimicrobial activity at 100 μg/ml. It has low toxicity; there were no deaths after ip injection of mice with 250 mg/kg.

Nagstatin, a strong inhibitor against NAG-ase, potentiates cellular immune response in normal mice and also reactivates the depressed response in tumor bearing mice. Its potentiating activity is almost equal to that of bestatin[14]. Details on immunological properties of nagstatin will be described in another paper.

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