BENARTHIN: A NEW INHIBITOR OF PYROGLUTAMYL PEPTIDASE

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

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We found benarthin, a new inhibitor of pyroglutamyl peptidase, in the fermentation broth of *Streptomyces xanthophaeus* MJ244-SF1. It was purified by column chromatography and centrifugal partition chromatography (CPC) and then was isolated as a colorless powder. The binding of benarthin was competitive with substrate and its inhibition constant (*Ki*) was 1.2×10^{-6} M.

Pyroglutamyl peptidase (PG-peptidase, EC 3.4.19.3) was first discovered in 1968 by DOOLITTLE and ARMENTROUT in *Pseudomonas fluorescens*[1]. The enzyme specifically releases proglutamyl residues which block terminal amino acids in many proteins and peptides such as thyrotropin-releasing hormone[2,3] (TRH), luteinizing hormone-releasing hormone[4] (LH-RH), neurotensin[5] and bombesin[5]. PG-peptidase is now known to be widely distributed in various animal tissues[6], bacteria[7] and plants[6]. However, little information has been available concerning the physiological function of the enzyme. Its physiological role is attracting interest among many researchers. In order to elucidate the relationship between various disease processes and the enzyme, we searched for specific inhibitors of PG-peptidase.

In the course of screening for inhibitors of PG-peptidase, we discovered benarthin as a specific inhibitor. In this communication we report the taxonomy of the producing organism, as well as the production, isolation and biological activities of benarthin.

Materials and Methods

General

Chemicals employed were as follows: L-Pyrogultamyl-β-naphthylamide (PG·NA) from Bachem Feichemikalien AG, Budendorf, Switzerland, and Fast garnet GBC (*o*-aminoazotoluene, diazonium salt) from Sigma Chemical Co., St. Louis, U.S.A.

Enzyme

Bovine (calf) liver PG-peptidase (EC 3.4.19.3) was purchased from Sigma Chemical Co., St. Louis, U.S.A.

Microorganism

Strain MJ 244-SF1 was isolated from a soil sample collected on the premises of the Institute of Microbial Chemistry, Shinagawa-ku, Tokyo and was deposited in the Fermentation Research Institute, Agency of Industrial Science under the accession No. FERM P-11769.

Taxonomic Characterization

Morphological and physiological characteristics of strain MJ244-SF1 were examined according to the methods described by SHIRLING and GOTTLIEB[8], and WAKSMAN[9].

Fermentation of Benarthin

Strain MJ244-SF1 was stored and maintained on slants of yeast starch (YS) agar consisting of soluble starch 1.0%, yeast extract 0.2%, and Difco Bacto agar 1.7%, pH 7.0. A loopful of mycelial suspension from a slant culture of strain MJ244-SF1 incubated for 10 days was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of a medium consisting of galactose 2.0%, dextrin 2.0%, Bacto-Soytone (Difco) 1.0%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, and $CaCO_3$ 0.2% (adjusted to pH 7.4 using 6 N NaOH before sterilization, 120°C, 20 minutes). The inoculated flask was incubated at 27°C for 24 hours on a rotary shaker (180 rpm). Two ml of the seed culture were transferred to 110 ml of the same medium in a 500-ml Erlenmeyer flask and were cultured for approximately 48 hours under the same conditions.

Isolation of Benarthin

The purification procedures for benarthin are shown in Scheme 1. After 2 days of incubation at 27°C, the fermentation broth was separated from the mycelium by filtration. The inhibitor in the broth filtrate (4 liters) was adsorbed onto an activated carbon column (500 ml). The column was washed with water and was eluted with 60% aq Me_2CO (pH 9.0). The active fractions were pooled and concentrated under reduced pressure to remove Me_2CO and the solution containing benarthin was adsorbed onto a column of Diaion HP-20 (Mitsubishi Chemical Industries Limited) (200 ml). The column was washed with water and was eluted with 50% aq Me_2CO. The eluate fractions containing benarthin were concentrated under reduced pressure to give a crude powder (2.85 g). A solution of this crude powder in water was chromatographed using a Sephadex G-10 column. The active fractions were concentrated to give a crude powder (500 mg).

The crude powder was subjected to CPC. The chromatography was performed using a CPC apparatus model NMF (Sanki Engineering Limited) employing the following conditions: BuOH - AcOH - H_2O (upper phase stationary, 750 : 50 : 750), 4 ml/minute, 1,000 rpm, 20°C, detection 254 nm, 6 ml fractions. The fractions containing benarthin were collected and concentrated to a small volume and were lyophilized to give a colorless powder of benarthin acetate (396 mg).

In order to obtain the benarthin HCl salt for elemental analysis, benarthin (30 mg) was dissolved in 0.1 N HCl (3 ml) and the solution was lyophilized to give the benarthin HCl salt as a colorless powder (28 mg).

Assay for PG-peptidase and Inhibitory Activity

PG-peptidase activity was assayed by a modification of the method of EXTERKATE[10]. The reaction mixture (total 0.2 ml) contained 0.1 ml of phosphate buffer pH 7.6 (0.2 M K_2HPO_4 buffer - 0.1 M EDTA - 0.01 M dithiothreitol, 5 : 1 : 1), 0.02 ml of 5 mM PG·NA and 0.06 ml of water or aqueous solution containing the test compound. The mixture was incubated at 37°C for 3 minutes and 0.02 ml of PG-peptidase solution (0.17 U) was added. The reaction mixture was incubated at 37°C for 60 minutes and was treated with 0.01 ml of 16 mM $NaIO_4$ solution. After 15 minutes, the enzymatic reaction was terminated by the addition of 0.1 ml of 1 M sodium citrate buffer solution (pH 4.0) containing 10% Tween 20 and 0.2% Fast garnet GBC. After allowing the mixture to stand at room temperature for 15 minutes, the absorbance at 525 nm was measured.

The percent inhibition was calculated by the formula $(A-B)/A \times 100$, 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 at 50% inhibition of enzyme activity.

Results and Discussion

Taxonomy of the Producing Organism

Taxonomic features of strain MJ244-SF1 are shown in Table 1. Strain MJ244-SF1 has straight aerial mycelia, forming chains of spores with more than 50 spores per chain. The spores are 0.6×0.7 by 1.2×1.3 μm

Table 1. Cultural characteristics of strain MJ244-SF1.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Pale yellow	Light brownish gray	None
Yeast extract-malt extract agar (ISP No. 2)	Dull yellow	Light brownish gray	Yellow
Oatmeal agar (ISP No. 3)	Pale yellow	Light brownish gray	Pale yellow
Inorganic salts-starch agar (ISP No. 4)	Pale yellow	Light gray	Faint yellowish
Glycerol-asparagine agar (ISP No. 5)	Yellow	Light gray	Yellow
Glucose-asparagine agar	Yellow	Light gray	Faint yellowish
Tyrosine agar (ISP No. 7)	Pale brown	Light gray~ light brownish gray	None
Nutrient agar	Pale brown	None	None

The color names used in this table were based on the Color Standard (Nihon Shinkisai Co., Ltd.).

Table 2. Comparison of taxonomic characteristics of strain MJ244-SF1 and *Streptomyces xanthophaeus*.

	MJ244-SF1	*S. xanthophaeus* IMC S-0121 (ISP5134)	*S. xanthophaeus*[9,11]
Spore chain morphology	*Rectiflexibiles*	*Rectiflexibiles*	*Rectiflexibiles*
Spore surface	Smooth	Smooth	Smooth
Aerial mass color	Bright brownish gray to bright gray	Bright brownish gray	Bright brownish gray to bright gray
Color of vegetative growth	Pale yellowish brown to yellow	Pale yellowish brown	Pale yellow to brown
Soluble pigment	Yellow	White to pale yellow	Yellow
Melanin formation:			
ISP-medium 1	Positive	Positive	Positive
ISP-medium 6	Positive	Positive	Positive
ISP-medium 7	Positive	Positive	ND
Hydrolysis of starch	Strong	Strong	Strong
Coagulation of skim milk	Positive	Negative	Positive
Peptonization of skim milk	Positive	Negative	Positive
Liquefaction of gelatin:			
Plain gelatin	Negative	Negative	Positive
Glucose-peptone-gelatin	Negative	Negative	Positive
Nitrate reduction	Positive	Positive	ND
Carbon utilization:			
Glucose	+	+	+
L-Arabinose	−	−	−
D-Xylose	−	−	−
D-Fructose	−	−	−
Sucrose	−	−	−
Inositol	−	−	−
Rhamnose	−	−	−
Raffinose	−	−	−
D-Mannitol	−	−	−

ND: Not data, +: utlization, −: no utilization.

in size with smooth surfaces. No spiral formations or whirl-formations were observed. Soluble pigments were yellowish and were mixed with melanoid pigments. The whole-cell hydrolysate of the strain was found to contain L,L-diaminopimelic acid.

On the basis of its characteristics, strain MJ244-SF1 was considered to belong to the genus *Streptomyces*. Among the known species of *Streptomyces*, *S. xanthophaeus* is considered to be most similar to the strain

MJ244-SF1. The comparison of strain MJ244-SF1 and *S. xanthophaeus* is summarized in Table 2. As is apparent from data presented in Table 2, the taxonomic features of *S. xanthophaeus* IMC S-0121 were very similar to those of strain MJ244-SF2 except for the coagulation and peptonization of skim milk. Moreover, the published descriptions of *S. xanthophaeus*[9,11] are similar to those of MJ244-SF1. Therefore, strain MJ244-SF1 was classified and designated as *S. xanthophaeus* MJ244-SF1.

Fermentation and Isolation

S. xanthophaeus MJ244-SF1 was cultured in Erlenmeyer flasks at 27°C for 2 days on a rotary shaker. Growth, pH and consumption of carbohydrates are plotted in a time course study of the fermentation (Fig. 1). The maximum peak of benarthin production in the flasks was obtained at 2 days, thereafter the production decreased with a pH change to alkaline. The flow diagram for the isolation is shown in Scheme 1. The yield of pure benarthin acetate was 396 mg from 4 liters of broth filtrate. Production and isolation of benarthin were monitored by its inhibitory activity against PG-peptidase and by HPLC under the following conditions: Column; Capcell pak C_{18} (4.6 × 150 mm, Shiseido Co., Ltd.), solvent; 8% aq MeCN containing 0.5% AcOH, detection; UV at 254 nm, flow rate; 1 ml/minute. The physicochemical properties and determination of the chemical structure of benarthin are reported in a companion paper[12].

Scheme 1. Isolation of benarthin.

Streptomyces xanthophaeus MJ244-SF1

- Broth filtrate (4 liters, IC_{50} = 5 μl/ml)
  - Carbon
    - eluted with 50% $(CH_3)_2CO$(pH 9.5)
  - Diaion HP-20
    - eluted with 50% $(CH_3)_2CO$
  - Sephadex G-10
    - eluted with H_2O
  - Centrifungal partition chromatograph
    - solvent system: *n*-BuOH - AcOH - H_2O
  - Benarthin (colorless powder) 396 mg, IC_{50} = 2 μg/ml
- Mycelia

Fig. 1. Time course of benarthin production by *Streptomyces xanthophaeus* MJ244-SF1.

○ Potency, △ pH, □ galactose.

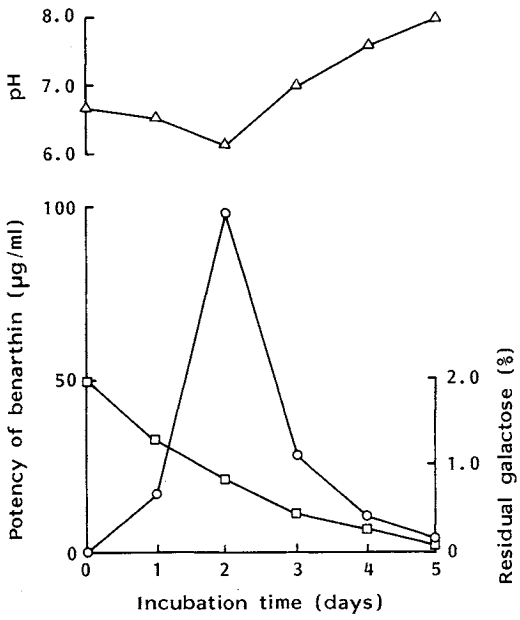
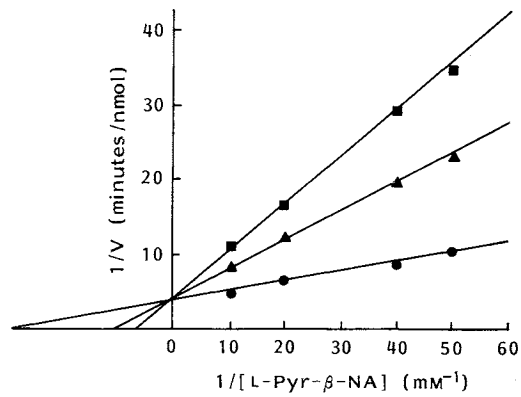


Fig. 2. Lineweaver-Burk plot of inhibition of pyroglutamyl peptidase by benarthin.

■ I = 2 μg/ml, ▲ I = 1 μg/ml, ● I = 0 μg/ml. $Km = 3.3 \times 10^{-5}$ M, $Ki = 1.2 \times 10^{-6}$ M.



Biological Activities

The inhibitory activity (IC_{50}) of benarthin was determined to be 2.0 μg/ml against PG-peptidase. As shown in Fig. 2, the inhibition of benarthin against PG-peptidase was competitive with substrate and the *Ki* and *Km* values were 1.2×10^{-6} M and 3.3×10^{-5} M, respectively[12]. It had no antimicrobial activity at 100 μg/ml. Benarthin had a low toxicity; no deaths occurred after its intravenous injection at 100 mg/kg to mice.

Acknowledgments

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