EURYSTATINS A AND B, NEW PROLYL ENDOPEPTIDASE INHIBITORS†

I. TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL ACTIVITIES

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Eurystatins A and B, were isolated from the cultured broth of *Streptomyces eurythermus* R353-21. They showed specific and potent inhibitory activity against prolyl endopeptidase and did not show antimicrobial activity. No lethal toxicity was observed for the two compounds after ip administration in mice at 200 mg/kg.

Proteolytic enzymes are actively involved in every phase of anabolism and catabolism in all sorts of plants and animals. Accordingly, specific protease inhibitors are highly probable to find therapeutic utilities in the clinical treatment of various diseases such as ALZHEIMER's disease, thrombosis, AIDS and cancer. Prolyl endopeptidase (PED), a serine protease, which is known to catalyze the hydrolysis of several biologically active peptides such as oxytocin, vasopressin, angiotensins I and II, substance P, bradykinin, and neurotensin at the carboxyl side of their proline residue, is considered to play an important role in the biological regulation of these peptides. D. DE WIED and coworkers suggested[1] that vasopressin might be involved in the process of learning and memory of animals. Recently, AOYAGI *et al.* have observed[2] that PED activity was pathogenetically elevated in the brain of patients with ALZHEIMER's disease. In addition, several inhibitors of this enzyme have been reported to have anti-amnesic activities in animal models[3,4]. Such information encouraged us to screen for new PED inhibitors from microbial metabolites.

In the course of our systematic screening for PED inhibitors, *Streptomyces eurythermus* R353-21, was found to produce a complex of novel PED inhibitors designated as eurystatins A and B. In this paper, the taxonomy of the producing strain, and production, isolation and biological activities of eurystatins A and B are presented. Physico-chemical properties and structural studies will be reported in the accompanying paper[5].

Materials and Methods

Taxonomic Studies

Strain R353-21 was isolated from a soil sample collected in India. Morphological, cultural and physiological characterization of the strain was carried out by the methods of the International Streptomyces Project (ISP)[6], and by some other supplementary tests. The whole cell diamino acid and phospholipid compositions were analyzed by the methods of LECHEVALIER[7] and LECHEVALIER *et al.*[8], respectively.

Fermentation

A loopful of the slant culture of strain R353-21 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of vegetative medium consisting of soluble starch (Nichiden Kagaku) 3%, Bacto-liver

† Eurystatins A and B were originally called BU-4164E A and BU-4164E B, respectively.

(Difco) 1%, Polypepton (Daigo Eiyo Kagaku) 0.5%, NaCl 0.3%, $(NH_4)_2SO_4$ 0.1% and $CaCO_3$ 0.6%, pH being adjusted to 7.0 before autoclaving. After incubated at 28°C for 3 days on a rotary shaker (200 rpm), 5 ml of the growth was transferred to a 500-ml Erlenmeyer flask containing 100 ml of production medium having the same composition as the vegetative medium. The fermentation was carried out at 28°C for 4 days on a rotary shaker (200 rpm). The resulting seed culture was inoculated to 120 liters of production medium containing the same composition as the seed medium in a 200-liter tank fermenter and cultured at 28°C for 3 days with agitation at 250 rpm and aeration of 120 liters per minute.

Assay of Prolyl Endopeptidase Inhibitory Activity

Inhibitory effects of eurystatins A and B on prolyl endopeptidase (PED) were determined by *Flabobacterium*[9] and partially purified rabbit brain enzymes according to the procedure of YAMAKAWA *et al.*[10]. Ten μl of *Flavobacterium meningosepticum* PED (Seikagaku Kogyo Co. Ltd., 0.5 unit/ml in 50 mM phosphate buffer, pH 7.0) or rabbit brain PED, a test sample solution (13.5 μl) and 100 mM phosphate buffer, pH 7.0 (86.5 μl) were mixed in a well of a 96-well microplate and pre-incubated for 30 minutes at 30°C. The reaction was started by addition of 25 μl of 2 mM carbobenzyloxyglycyl-L-prolyl-*p*-nitroanilide in 40% dioxane - 100 mM phosphate buffer, pH 7.0 as substrate. After incubation at 30°C for 5 minutes, the amount of released *p*-nitroaniline was determined colorimetrically with a microplate autoreader at 414 nm. Carbobenzyloxy-L-valyl-prolinal (Z-val-prolinal), a synthetic PED inhibitor, was used as a reference compound.

Antimicrobial Activity

The antimicrobial activities of eurystatins A and B were determined by the 2-fold agar dilution method using nutrient agar (Eiken) for 23 strains of Gram-positive and Gram-negative bacteria, and Sabouraud dextrose agar for 14 strains of fungi. The antimicrobial activity was determined after 18 ~ 20 hours incubation at 37°C for bacteria and after 40 ~ 60 hours incubation at 28°C for fungi.

Isolation and Purification of Eurystatins A and B

The harvested whole broth (220 liters) was extracted under stirring with BuOH (96 liters). The organic layer (80 liters) was separated with a Sharples type centrifuge (Kokusan No. 4A) and evaporated to dryness under reduced pressure. The residue was applied on a column of Diaion HP-20 (Mitubishi Chem. Ind., 2 liters), and developed with water (6 liters), 50% aqueous MeOH (7 liters), 30% aqueous acetonitrile (MeCN, 6 liters), and 40% aqueous MeCN (4 liters), successively. The eluate after 50% aqueous MeOH was collected in 120-ml fractions and monitored for PED inhibitory activities. Active fractions were combined and evaporated until the MeCN was removed. The aqueous solution was extracted with EtOAc (1.5 liters × 2). The extracts were combined and concentrated to dryness to give 4.48 g of a brown powder. This powder was chromatographed on a column of silica gel (Wako gel C-300, 700 ml) with CH_2Cl_2 - EtOAc - MeOH (100 : 4 : 2, 1.5 liters) and (100 : 6 : 3, 3.5 liters), successively. The eluate was monitored by the enzyme inhibitory activity assay and TLC (SiO_2; CH_2Cl_2 - EtOAc - MeOH (10 : 3 : 1)). Active fractions were combined and evaporated under reduced pressure to afford 329 mg of white amorphous powder whose HPLC analysis showed the presence of eurystatins A and B as shown in Fig. 2. A 260 mg amount of this powder was chromatographed on a reversed phase silica gel column (YMC GEL A60; Yamamura Chemical Lab., 700 ml) with MeCN - 0.022 M phosphate buffer (pH 7.0) (30 : 70, 2 liters), (35 : 65, 2 liters) and (40 : 60, 1 liter), successively, under monitoring by TLC (SiO_2; CH_2Cl_2 - EtOAc - MeOH (10 : 3 : 1)) and HPLC (column; YMC-Pack (A-301-3) 4.6 mm i.d. × 100 mm, Yamamura Chemical Lab., mobile phase; MeCN - 0.022 M phosphate buffer (pH 7.0) (35 : 65), flow rate; 1 ml/minute, detection; UV absorption at 210 nm).

Fractions containing eurystatin A, which showed a retention time of 3.8 minutes on HPLC, were combined and evaporated under reduced pressure until the organic solvent was removed. The residual aqueous phase was extracted with BuOH. The BuOH extract was evaporated and lyophilized to give 157 mg of a pure white solid of eurystatin A. This solid was subjected to crystallization in aqueous MeCN to afford colorless fine needles (85 mg). Fractions containing eurystatin B, which showed a retention time of 6.2 minutes on HPLC, were combined and similarly treated to afford 86 mg of pure eurystatin B. The product was also subjected to crystallization in aqueous MeCN to give fine needles (46 mg).

Results

Taxonomy of the Producing Strain

Strain R353-21 formed both substrate and aerial mycelia. Spore chains which were born monopodially or in tuft on aerial mycelia, were open spiral, loop or straight, and contained 10 to 30 or more spores per chain. The spore chain morphology belonged to *Retinaculiaperti*. Scanning electron micrography indicated that the spores were spherial to oval ($0.7 \sim 1.0 \times 0.7 \sim 1.5\ \mu m$) with smooth surface.

The cultural characteristics and the carbohydrate utilization profile of strain R353-21 are shown in Tables 1 and 2, respectively. The color of aerial mass was gray with shades of yellow or brown (Gray color-series). Melanin was produced in ISP No. 1 broth and ISP No. 6 agar. LL-Diaminopimelic acid was detected in the whole cell hydrolysate. Phospholipids contained phosphatidylethanolamine, phosphatidylinositol mannoside and phosphatidylinositol. Hence, the strain belongs to cell-wall Type I and phospholipid Type P-II. Based on the taxonomic properties as described above, strain R353-21 was classified in the genus *Streptomyces* Waksman and Henrici 1943. According to the descriptions of PRIDHAM and TRESNER[11], and SHIRLING and GOTTLIEB[12], strain R353-21 was considered to resemble *Streptomyces eurythermus* CORBAZ *et al.*[12]. Subsequent direct comparison with *S. eurythermus* IFO12764^T revealed that strain R353-21 differed from strain IFO12764 only in the growth on CZAPEK's sucrose-nitrate agar. Thus, strain R353-21 was identified as a strain of *S. eurythermus*, and designated *Streptomyces eurythermus* R353-21. A culture of this strain has been deposited in American Type Culture Collection, U.S.A., under accession No. ATCC 55001.

Table 1. Cultural characteristics of strain R353-21.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-DOX agar)	Moderate	Abundant; medium gray (265)	Grayish yellowish brown (80)	None
Tryptone-yeast extract broth (ISP No. 1)	Abundant; floccose, not turbid	None	Colorless	Moderate brown (58)
Yeast extract-malt extract agar (ISP No. 2)	Moderate	Moderate; olive gray (113)	Colorless	Moderate yellowish brown (77)
Oatmeal agar (ISP No. 3)	Moderate	Moderate; white to light olive gray (112)	Colorless	None
Inorganic salts-starch agar (ISP No. 4)	Abundant	Abundant; olive gray (113)	Dark gray (266)	None
Glycerol-asparagine agar (ISP No. 5)	Moderate	Poor, light olive gray (112)	Light grayish yellowish brown (79)	Light grayish yellowish brown (79)
Peptone-yeast extract-iron agar (ISP No. 6)	Abundant	None	Colorless	Moderate brown (58)
Tyrosine agar (ISP No. 7)	Moderate	None or scant; white	Light grayish yellowish brown (79)	Light brown (57)
Glucose-asparagine agar	Poor	None	Colorless	None
Nutrient agar	Moderate	None	Colorless	None

Observation after incubation at 28°C for 3 weeks.
Color and number in parenthesis follows ISCC-NBS designation.

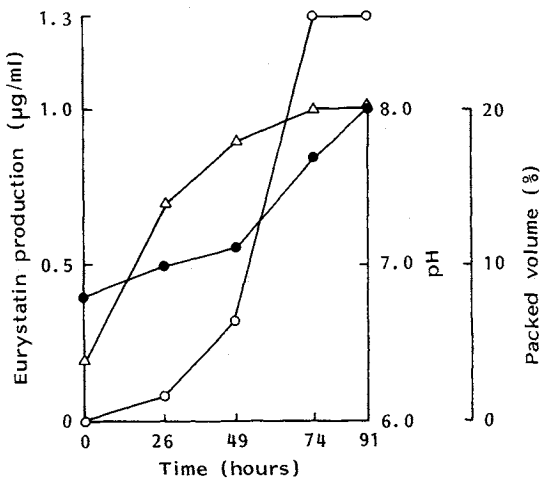
Table 2. Physiological characteristics of strain R353-21.

Hydrolysis of:		D-Xylose	+
Gelatin	+	D-Ribose	+
Starch	+	L-Rhamnose	+
Milk coagulation	+	D-Glucose	+
Milk peptonization	+	D-Galactose	+
Production of:		D-Fructose	+
Nitrate reductase	− or +[a]	D-Mannose	+
Tyrosinase	+	L-Sorbose	−
Tolerance to:		Sucrose	+
Lysozyme 0.01% (w/v)	−	Lactose	+
0.001%	+	Cellobiose	+
NaCl 1~7% (w/v)	+	Melibiose	+
9%	−	Trehalose	+
pH	5.3~10.5	Raffinose	−
Temperature:		D-Melezitose	−
Growth range	14~14°C	Soluble starch	+
Optimal growth	35~41°C	Cellulose	−
No growth	11 and 47°C	Dulcitol	−
Utilization of[b]:		Inositol	+
Glycerol	+	D-Mannitol	+
D-Arabinose	−	D-Sorbitol	−
L-Arabinose	+	Salicin	±

[a] Negative in peptone - nitrate broth and positive in CZAPEK's sucrose - nitrate broth.
[b] Basal medium: PRIDHAM-GOTTLIEB's inorganic salts medium (ISP No. 9).

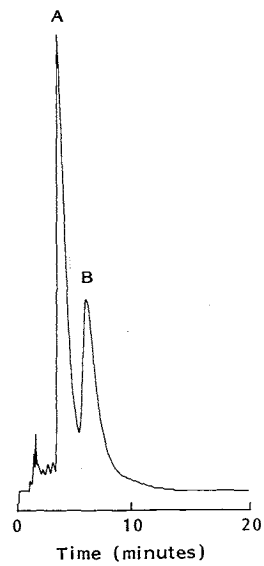
Fig. 1. Time course of eurystatin production by *Streptomyces eurythermus* R353-21.

○ Eurystatin production, ● pH, △ packed volume.



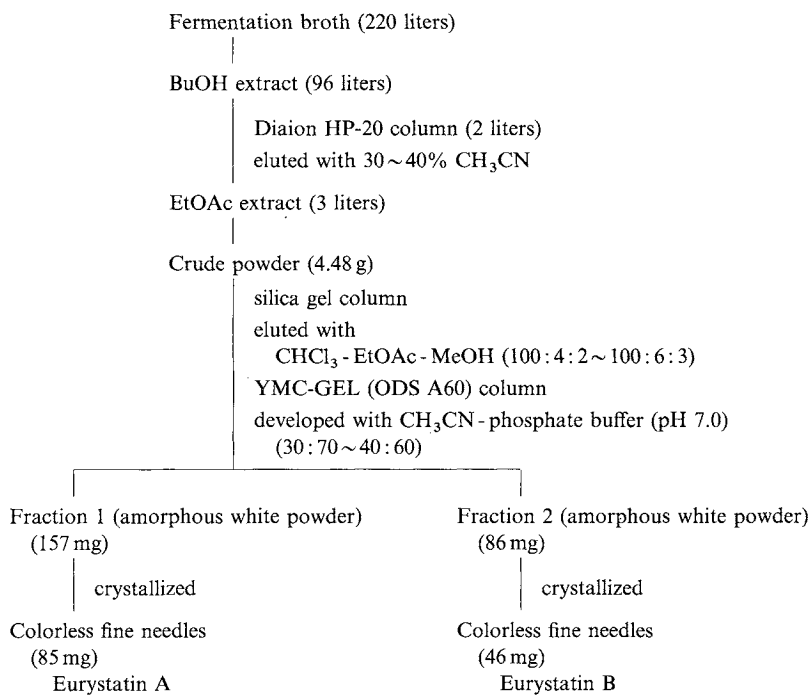
Production of eurystatins A and B was collectively calculated as eurystatin A based on PED inhibition potency of broth.

Fig. 2. HPLC chromatogram of eurystatins A and B.



Column: YMC-Pack (A-301-3) 4.6 mm i.d. × 100 mm, 3 μm, ODS. (Yamamura Chemical Lab.)
Mobile phase: CH_3CN - 0.022 M phosphate buffer (35 : 65, pH 7.0)
Flow rate: 1 ml/minute.
Detection: UV absorption at 210 nm.

Fig. 3. Isolation and purification procedure of eurystatins A and B.



Production and Purification

A large scale fermentation was carried out by transferring 2 liters of the flask seed culture to a 200-liter tank fermenter containing 120 liters of the production medium at 28°C for 3 days. The eurystatin production in fermentation broth was monitored by the *Flavobacterium* PED inhibition assay. The time course of eurystatins A and B fermentation is shown in Fig. 1. Eurystatin production reached a plateau after 3 days of cultivation and the fermentation yield was 1.3 μg/ml. The isolation procedure is shown in Fig. 3 and in Material and Method section. The IR spectra suggested eurystatins A and B to be peptides, and acid hydrolysis of them gave ninhydrin positive spots on TLC. Detail of the structure determination of these compounds is reported in the companion paper[5].

Table 3. Inhibitory effects of eurystatins A and B and Z-val-prolinal on *Flavobacterium* and rabbit brain prolyl endopeptidases.

Compound	IC_{50} (μg/ml)	
	Flavobacterium PED	Rabbit brain PED
Eurystatin A	3.7	85
Eurystatin B	2.1	31
Z-Val-prolinal	3.6	16

Biological Activity

As shown in Table 3, both eurystatins A and B strongly inhibited both the enzymes with IC_{50} values of 3.7 and 2.1 ng/ml against *Flavobacterium* PED, and of 85 and 31 ng/ml against rabbit brain PED, respectively. By using a partially purified enzyme, the rabbit brain PED was significantly less inhibited by eurystatins A and B than by synthetic inhibitor, Z-val-prolinal.

Neither eurystatins A nor B showed antimicrobial activities at 100 μg/ml against the bacteria and fungi tested.

When 5 male IRC mice weighing about 25 g were intraperitoneally administered with 200 mg/kg of eurystatin A or B, neither lethal toxicity nor behavioral effect was seen during an observation period of 10 days.

Discussion

Our systematic search for new PED inhibitors resulted in the discovery of eurystatins A and B from the cultured broth of *S. eurythermus* R353-21. They showed potent inhibitory activities against *Flavobacterium* PED comparable to that of Z-val-prolinal. Most of PED inhibitors reported so far are the synthetic pyrrolidine derivatives[3,13] including Z-val-prolinal or endogenus high molecular weight substances[14,15]. The synthetic inhibitors have an aldehyde moiety in their *C*-terminal which is considered to be the key functional group for the activity. However, poststatin, a new PED inhibitor was recently discovered from cultured broth of *Streptomyces viridochromogenes* MH534-30F3, and shown to have potent and specific PED inhibitory activity[16]. It is interesting that poststatin is a linear peptide having a unique of α-keto-β-amide group[17]. The modification studies of poststatin revealed that the α-keto functionality was essential for the PED activity. Eurystatins are new addition to the PED inhibitors having the interesting α-keto-β-amide functionality. The specific and potent PED inhibitory activities of eurystatins suggest a possibility for good anti-amnesic activities without toxicity.

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