VOL. 54 NO. 12, DEC. 2001 THE JOURNAL OF ANTIBIOTICS pp. 997 — 1003

Tyropeptins A and B, New Proteasome Inhibitors Produced by *Kitasatospora* sp. MK993-dF2

I. Taxonomy, Isolation, Physico-chemical Properties and Biological Activities

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(Received for publication July 23, 2001)

Tyropeptins A and B, new proteasome inhibitors, were isolated from the culture broth of *Kitasatospora* sp. MK993-dF2. They were purified using ethyl acetate extraction, silica gel column chromatography, Sephadex LH-20 column chromatography and HPLC. Tyropeptin A inhibited the chymotrypsin-like (ChT-L) and trypsin-like (T-L) activities of 20S proteasome with IC_{50} values of 0.1 μg/ml and 1.5 μg/ml respectively, but did not inhibit the peptidylglutamyl-peptide hydrolyzing (PGPH) activity of 20S proteasome at a concentration of 100 μg/ml. The inhibitory activities of tyropeptin A were about two times as strong as that of tyropeptin B. Taxonomy of the producing strain is also described.

The ubiquitin-proteasome pathway is the principle pathway for intracellular protein degradation in both the nucleus and cytosol[1]. Protein substrates are marked with a poly-ubiquitin chain[2] and then degraded to peptides and free ubiquitin by a high molecular weight multicatalytic protease complex, the 26S proteasome, which exists within all eukaryotic cells[1]. This pathway is involved in many biological processes, including the removal of abnormal, misfolded or improperly assembled proteins, stress response, cell-cycle control, cell differentiation and metabolic adaptations and the cellular immune response (by generating antigenic peptides presented by major histocompatibility complex (MHC) class I molecules).

The catalytic core of 26S proteasome is the 20S proteasome, which is a large cylindrically shaped complex composed of a stack of four rings, each containing seven subunits, $\alpha_7\beta_7\beta_7\alpha_7$[3]. The 20S proteasome displays multicatalytic activities, and at least three distinctive activities, ChT-L (Chymotrypsin-like), T-L (Trypsin-like) and PGPH (Peptidylglutamyl-peptide hydrolyzing) are observed. Three distinct β-type subunits, each with an *N*-terminal Thr^1 active site, are responsible for ChT-L, T-L and PGPH activities of the 20S proteasome.

Numerous regulatory proteins have been found to undergo ubiquitin dependent proteolysis[4]. Many of these proteins function as important regulators of physiological or pathophysiological cellular processes. Importantly, The ubiquitin-proteasome pathway plays a critical role in tumor growth and inflammatory process. Cyclins, CDK inhibitors (*e.g.* p21, p27) and tumor suppressors (*e.g.* p53) are degraded by this pathway[5~7]. In addition, the proteasome is required for activation of NF-κB through degradation of its inhibitory protein, IκB[8]. NF-κB is a key transcription factor in inflammation which is required for the expression of many proinflammatory mediators. Thus, proteasome inhibitors may be promising new therapeutics for treatment of cancer and inflammatory diseases.

In the course of our screening work for new proteasome inhibitors, we found new peptidyl aldehyde proteasome inhibitors, tyropeptins A and B, which were produced by

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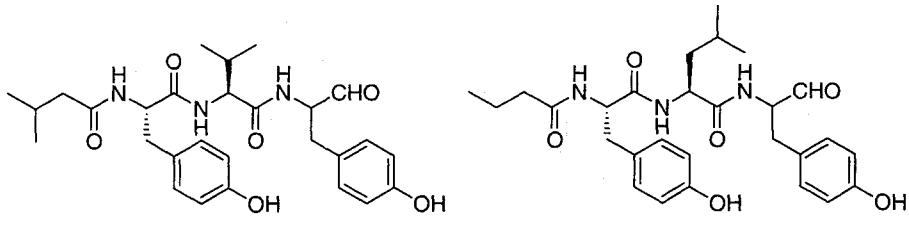
Fig. 1. The structures of tyropeptins A and B.



Tyropeptin A Tyropeptin B

Kitasatospora sp. MK993-dF2 (Fig. 1). In this paper, the taxonomy of the producing strain, isolation, physicochemical properties and biological activities of tyropeptins A and B are reported. The structural studies of tyropeptins A and B will be described in the following paper[9].

Materials and Methods

Materials

α-Chymotrypsin and m-calpain were purchased from Sigma Chemical Co. Cathepsin L was purchased from Calbiochem Corp. The inhibitor (MG-132) and substrates (Suc-Leu-Leu-Val-Tyr-MCA, Boc-Leu-Arg-Arg-MCA, *Z*-Leu-Leu-Glu-MCA and *Z*-Phe-Arg-MCA) for protease were purchased from Peptide Institute Inc. Lactacystin was purchased from Kyowa Medex Co., Ltd.

Microorganism

The tyropeptins A and B producing strain, MK993-dF2 was isolated from a soil sample collected at Kami-gun, Miyagi prefecture, Japan. This strain has been deposited in the National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan under the accession number FERM P-18233.

Taxonomic Studies

Morphological characteristics of the spores and mycelia were observed with a scanning electron microscope (Hitachi S-570). Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTILIEB[10], and WAKSMAN[11]. The substrate and aerial mycelium color including soluble pigments were assigned by the Color Harmony Manual, 1958 (Container Corporation of America, Chicago). Carbohydrate utilization was investigated using the procedure of PRIDHAM and GOTTLIEB[12]. Whole-cell sugars were determined by the method described previously[13]. 2,6-Diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[14] and STANECK and ROBERTS[15]. Phospholipids were analyzed by the procedure of MINNIKIN *et al.*[16]. Menaquinones were analyzed using HPLC and mass spectrometry as described by TAMAOKA *et al.*[17]. Cellular fatty acids were prepared and determined by the method of SUZUKI and KOMAGATA[18]. DNA analysis were performed by the method reported previously[19].

Production of Tyropeptins A and B

A slant culture of strain MK993-dF2 was inoculated into a 500-ml Erlenmeyer flask containing 110 ml of preculture medium containing galactose 2.0%, dextrin 2.0%, Bacto-soytone (Difco) 1.0%, corn steep liquor 0.5%, glycerol 2.0%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and a drop of silicone oil (Shin-Estu Chemical Industry, KM-70) (pH 7.4 before sterilization). The flask was shaken on a rotary shaker (180 rpm) at 30°C for 72 hours.

This preculture (3 ml) was transferred into 500-ml Sakaguchi flasks each containing 125 ml of the production medium containing glycerol 2.0%, dextrin 2.0%, Bacto-soytone (Difco) 1.0%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and a drop of silicone oil (pH 7.4 before sterilization). Production of tyropeptins A and B were carried out on a reciprocal shaker for 96 hours at 27°C.

Preparation of Proteasome

Proteasome was prepared from mouse liver as described by UGAI *et al.*[20], with some modifications and simplification. Briefly, the livers from female mice (ICR)

were homogenized in 3 volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol (DTT), 2 mM ATP and 0.25 M sucrose. The homogenate was centrifuged for 1 hour at 35,000 rpm, and then the resulting supernatant was recentrifuged for 5 hours at 40,000 rpm. The precipitate was suspended in a suitable volume of 25 mM Tris-HCl buffer (pH 8.0) containing 1 mM DTT, 2 mM ATP and 20% glycerol, and then was centrifuged for 30 minutes at 13,000 rpm. The obtained supernatant was stored at −70°C and used as proteasome.

Enzyme Assays

Generally, the activities of proteases were measured by monitoring the increase in fluorescence ($EX_{380\,nm}/EM_{460\,nm}$) that accompanies the cleavage of 7-amino-4-methylcoumarin (AMC) from the substrates using the Cytofluor 2350 Fluoresence Measurement System (Millipore Co.). The reaction mixture in the volume of 90 μl was preincubated at 37°C for 5 minutes, and enzyme reaction was stopped by adding 50 μl of 10% SDS.

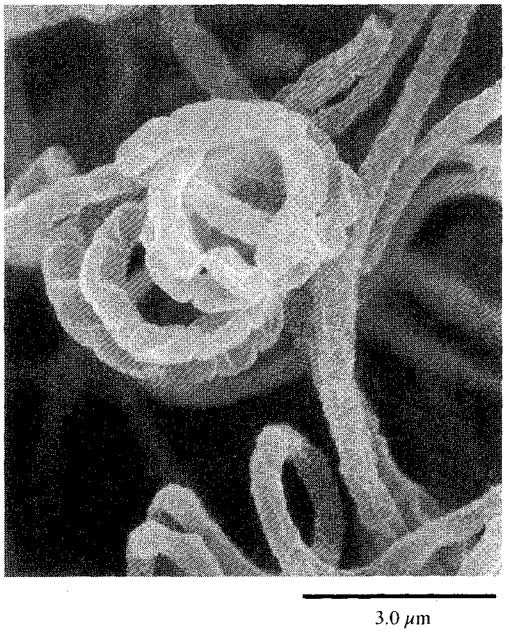
In the assay of proteasome, the 90 μl of reaction mixture contained 50 mM Tris-HCl buffer (pH 8.0), 1 mM DTT, 0.04% SDS, 0.11 mM substrate and the test material. The reaction was initiated by the addition of 10 μl of proteasome, and then incubated for 20 minutes. The ChT-L, T-L and PGPH activities of 20S proteasome were measured using Suc-Leu-Leu-Val-Tyr-MCA, Boc-Leu-Arg-Arg-MCA and *Z*-Leu-Leu-Glu-MCA as substrates, respectively.

In the assay of α-chymotrypsin, the 90 μl of reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 100 mM NaCl, 1 mM $CaCl_2$, 0.11 mM Suc-Leu-Leu-Val-Tyr-MCA and the test material. The reaction was initiated by the addition of 10 μl of α-chymotypsin, and then incubated for 30 minutes.

In the assay of m-calpain, the 90 μl of reaction mixture contained 100 mM Tris-HCl buffer (pH 7.4), 2 mM 2-mercaptoethanol, 2 mM $CaCl_2$, 0.11 mM Suc-Leu-Leu-Val-Tyr-MCA and the test material. The reaction was initiated by the addition of 10 μl of m-calpain, and then incubated for 15 minutes.

In the assay of cathepsin L, the 90 μl of reaction mixture contained 100 mM acetate buffer (pH 5.5), 1 mM EDTA, 8 mM L-cystein, 0.11 mM *Z*-Phe-Arg-MCA and the test material. The reaction was initiated by the addition of 10 μl of cathepsin L, and then incubated for 15 minutes.

Fig. 2. Scanning electron micrograph of strain MK993-dF2 on glucose-asparagine agar after incubation at 27°C for 10 days.



Results and Discussion

Taxonomy of the Tyropeptins A and B Producing Strain

Strain MK993-dF2 formed well-branched vegetative mycelia and aerial hyphae that bore spirals. The spores were cylindical (0.5~0.7×0.9~1.5 μm) with smooth surfaces as shown in Fig. 2. No synnemata, sclerotia, sporangia and motile spores were observed. Cultural characteristics of strain MK993-dF2 on various agar media are shown in Table 1. Physiological properties of strain MK993-dF2 are shown in Table 2. The permissive temperature range for growth was 10°C to 37°C, the optimal temperature range was 24°C to 30°C. The chemotaxonomic characteristics of strain MK993-dF2 are shown to the following. An analysis of cell wall hydrolysate revealed the presence of ribose, mannose, galactose and glucose as diagnostic sugars. 2,6-Diaminopimelic acid was present as both *meso*- and LL-isomers. These data indicated that strain MK993-dF2 had a type C whole-cell sugar pattern. The phospholipid type was PII, which contained phosphatidylethanolamine, but none of phosphatidylmethylethanolamine, phosphatidylcholine or unknown glucosamine-containing phospholipids. The

Table 1. Cultural characteristics of strain MK993-dF2.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Colorless	None	None
Yeast extract-malt extract agar (ISP No. 2)	Pale yellowish brown [2 gc, Bamboo ~ 2 ie, Lt Mustard Tan]	Grayish white ~ Light brownish gray [2 fe, Covert Gray]	Faint, brownish
Oatmeal agar (ISP No. 3)	Pale yellow [1 ca, Pale Yellow] ~ Dusty yellow [1 1/2 gc, Dusty Yellow]	Grayish white ~ Light gray [1 fe, Griege]	None
Inorganic salts-starch agar (ISP No. 4)	Pale yellowish brown [2 ie, Lt Mustard Tan] ~Dark brown [3 nl, Dk Brown]	Light gray [1 fe, Griege]	Faint, brownish
Glycerol-asparagine agar (ISP No. 5)	Dusty yellow [1 1/2 gc, Dusty Yellow] ~Pale olive [1 1/2 ie, Lt Olive]	Yellowish gray [1 cb, Parchment]	Faint, brownish
Tyrosine agar (ISP No. 7)	Pale yellwish brown [2 ie, Lt Mustard Tan]	Yellowish gray [1 1/2 ec, Putty] ~ Light brownish gray [2 fe, Covert Gray]	Faint, brownish

Observation after incubation at 27 ℃ for 21 days.

Table 2. Physiological characteristics of strain MK993-dF2.

Temperature range for growth	10~37℃
Optimum temperature	24~30℃
Formation of melanoid pigment	(-)
Hydrolysis of starch	+
Reduction of nitrate	-
Utilization of	
L-Arabinose	-
D-Fructose	-
D-Glucose	+
Inositol	-
D-Mannitol	(-)
Raffinose	-
Rhamnose	-
Sucrose	-
D-Xylose	(-)

+ : positive; (-): probably negative; - : negative

micolic acids were absent. The major menaquinone was MK-9 (H_6) and MK-9 (H_8). Cellular fatty acids consisted of 12-methyltetradecanoic acid (*ai*-15:0), hexadecanoic acid (16:0) and 13-methyltetradecanoic acid (*i*-15:0). G+C content of the DNA was 73.9 mol %. 16S rDNA sequences were determined for strain MK993-dF2 (positions 59~489 of *Escherichia coli* numbering system). The analysis of 16S rDNA sequences indicated that strain MK993-dF2 was closely related to members of the genus *Kitasatospora*[21, 22].

On the basis of these characteristics, strain MK993-dF2 had been classified as a member of the genus *Kitasatospora*.

Isolation

The culture broth (10 liters) was centrifuged to separate the supernatant and the mycelium cake. The supernatant (9.2 liters) was extracted with EtOAc (9.2 liters), and then the EtOAc extract was evaporated to dryness to give a brown oil (2.9 g). This oily substance was chromatographed on a silica gel column (Merck silica gel 60) and eluted stepwise with the mixtures of $CHCl_3$-MeOH (100:0, 50:1, and 10:1). The active fractions were eluted with the mixture of $CHCl_3$-MeOH (10:1), and the active fractions were collected and concentrated under reduced pressure to give a brown oil (1.6 g). This oily substance was

Table 3. Physico-chemical properties of tyropeptins A and B.

	Tyropeptin A	Tyropeptin B
Appearance	White powder	White powder
MP	100~102 ℃	91~94 ℃
$[\alpha]_D^{23}$	-15.1 ° (C 0.1, MeOH)	-14.6 ° (C 0.2, MeOH)
Molecular formula	$C_{28}H_{37}N_3O_6$	$C_{28}H_{37}N_3O_6$
APCI-MS (m/z)	512 $(M+H)^+$	512 $(M+H)^+$
	510 $(M-H)^-$	510 $(M-H)^-$
HRFAB-MS (m/z)		
Calcd:	512.2751 (as $C_{28}H_{38}N_3O_6$)	512.2757 (as $C_{28}H_{38}N_3O_6$)
Found:	512.2761 $(M+H)^+$	512.2761 $(M+H)^+$
UV λ_{max} nm, (log ε) in		
MeOH	225.2 (4.08), 277.6 (3.51)	225.0 (4.42), 277.2 (3.62)
MeOH-HCl	225.2 (4.07), 277.6 (3.52)	224.8 (4.41), 277.2 (3.61)
MeOH-NaOH	243.2 (4.07), 288.2 (3.52)	243.4 (4.34), 293.2 (3.64)
IR ν_{max} KBr cm^{-1}	3420, 2970, 1730, 1640,	3380, 2700, 1730, 1650,
	1520, 1440, 1370, 1230	1520, 1440, 1390, 1240
TLC (Rf value)[a]	0.24	0.24

[a] Silica gel TLC (Merck Art. 105715) : $CHCl_3$-MeOH (10 : 1)

chromatographed on a silica gel column (Merck silica gel 60) and eluted stepwise with the mixtures of toluene - acetone (5 : 1, 4 : 1, 3 : 1 and 2 : 1). The active fractions were eluted with the mixture of toluene - acetone (3 : 1), and were collected and concentrated under reduced pressure to give a brown paste (465.3 mg). The paste was applied to a Sephadex LH-20 column and developed with MeOH as the eluting solvent. The active fractions were concentrated to give yellow powder (305.8 mg). The powder was chromatographed using reverse phase HPLC (Shiseido, Capcell Pak UG, 2.0×25 cm) with a solvent of CH_3CN - H_2O (30 : 70) at flow rate of 10 ml/minute to give yellow powder (98.6 mg). The obtained substance was further chromatographed using reverse phase HPLC as described above with a solvent consisted of CH_3CN - H_2O (25 : 75) to give yellow powder (14.1 mg). Finally the crude tyropeptins were separately purified by reverse phase HPLC with a solvent of CH_3CN - 5 mM ammonium carbonate (40 : 60) to give 1.4 mg of tyropeptin A and 1.1 mg of tyropeptin B, respectively.

Physico-chemical Properties

Physico-chemical properties of tyropeptins A and B are shown in Table 3. The compounds were soluble in MeOH and DMSO, slightly soluble in $CHCl_3$, EtOAc and water, but insoluble in hexane and water. The molecular formula for tyropeptins A and B were found to be the same ($C_{28}H_{37}N_3O_6$) by HRFAB-MS. Tyropeptins A and B gave a positive color reaction with molybdophosphoric acid - sulfuric acid, Rydon-Smith, 2,4-dinitrophenylhydrazine, iron (III) chloride and anisaldehyde-sulfuric acid reagents, but the ninhydrin reagent gave no color.

Biological Activities

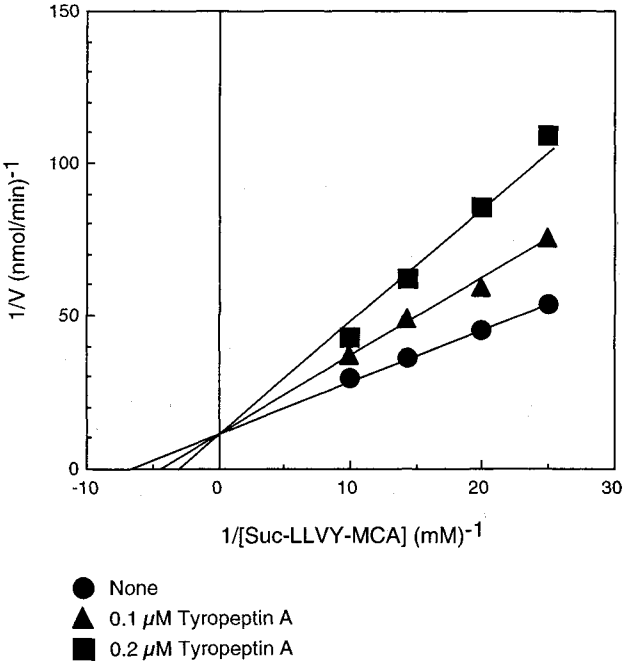
Enzyme inhibitory profiles of tyropeptins A and B are shown in Table 4. Tyropeptin A inhibited the ChT-L and T-L activities of 20S proteasome with IC_{50} values of 0.10 μg/ml and 1.5 μg/ml respectively, but did not inhibit the PGPH activity of 20S proteasome at a concentration of 100 μg/ml. The inhibitory activities of tyropeptin B against ChT-L and T-L activities of 20S proteasome were about one half of that of tyropeptin A. Lactacystin[23,24], a known proteasome inhibitor, inhibited the ChT-L activity with IC_{50} values of 1.5 μg/ml, but did not inhibit the PGPH activity at a concentration of 100 μg/ml. Kinetic studies of tyropeptin A were performed to determine the type of inhibition using

Table 4. Enzyme inhibitory profiles of tyropeptins A and B.

Enzyme		IC_{50} (μg/ml) Tyropeptin A	Tyropeptin B	MG-132
20S Proteasome	ChT-L	0.10	0.20	0.05
	PGPH	>100	>100	2.0
	T-L	1.5	4.0	2.0
α-Chymotrypsin		0.72	1.0	7.5
Cathepsin L		0.19	0.46	-
m-Calpain		0.74	0.54	-

- : Not determined

Fig. 3. Lineweaver-Burk plot for the ChT-L activity of 20S proteasome with tyropetin A.



substrate Suc-Leu-Leu-Val-Tyr-MCA. The results are demonstrated in Lineweaver-Burk (LB) plot with or without tyopeptin A as shown in Fig. 3. The inhibition of 20S proteasome by tyropeptin A was found to be of the competitive type, and *Km* value was 118 μM. *Ki* value of tyropeptin A was determined to be 0.22 μM by Dixon plot for the ChT-L activity of 20S proteasome.

Tyropeptin A showed cytotoxic activities against Hela S3 human cervical cancer cells and HL-60 human promyelocytic leukemia cells with IC_{50} values of 17.0 μg/ml and 4.4 μg/ml, respectively. The acute toxicity of tyropeptins A and B in mice (i.v.) were estimated to be quite low, with an LD_{50} value higher than 100 mg/kg.

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