YM-47141 and 47142, New Elastase Inhibitors Produced by *Flexibacter* sp. Q17897

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

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In the course of our screening for elastase inhibitors from microorganism, we have found two new cyclic-depsipeptides designated YM-47141 and 47142. In this paper, we describe the taxonomy of the producing organism and isolation, physico-chemical properties, and biological activities of YM-47141 and 47142.

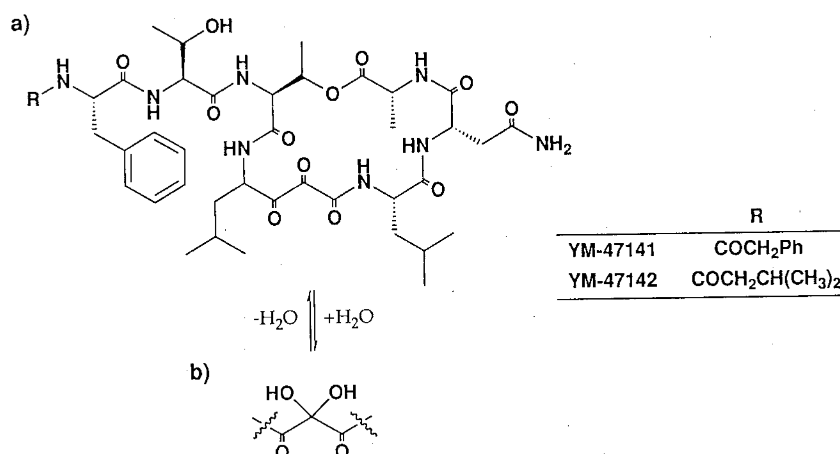
Human leucocyte elastase (HLE; EC 3.4.21.37) is a neutral serine proteinase, capable of degrading a variety of connective tissue components including elastin and collagen[1]. HLE has been implicated in the pathogenesis of a variety of inflammatory diseases such as emphysema, acute respiratory distress syndrome, and rheumatoid arthritis[2~4]. In most cases, the pathogenesis of these diseases has been ascribed to the inactivation or depletion of an endogenous HLE inhibitor, notably α_1-protease inhibitor. Therefore, inhibitors of HLE are potential therapeutic agents for these inflammatory diseases. In the course of our screening for elastase inhibitors from natural products, we have found two new depsipeptides designated YM-47141 and 47142 (Fig. 1) with activity against HLE. In this paper, we describe the taxonomy of the producing organism, isolation, physico-chemical properties, and biological activities of YM-47141 and 47142. The structure elucidation of YM-47141 and 47142 will be discussed in the following paper[5].

Experimental

Materials

HLE was purchased from Athens research and technology, Inc., U.S.A. Bovine plasma thrombin was

Fig. 1. Structures of YM-47141 and YM-47142.



obtained from Mochida Pharmaceutical Co., Ltd., Tokyo, Japan. Human plasma plasmin and synthetic substrate, S2222, S2238, S2251, S2302 were purchased from Daiichi pure chemicals, Tokyo, Japan. Bovine pancreatic trypsin, human plasma kallikrein, human leukocyte cathepsin G, bovine pancreatic α-chymotrypsin, and succinyl-Ala-Ala-Phe-Arg-*p*-nitroanilide were obtained from Sigma Chemicals Corp., St. Louis, U.S.A. Succinyl-Ala-Ala-Ala-*p*-nitroanilide was from Peptide Institute, Inc., Osaka, Japan.

Taxonomic Studies

The taxonomic studies were based on the methods described in BERGEY's Manual of Determinative Bacteriology 8th edition[6] and BERGEY's Manual of Systematic Bacteriology 9th edition[7]. Morphological observation of the strain Q17897 was carried out with a light microscope and a scanning electron microscope using the cells grown on nutrient agar for 120 hours at 33°C.

Fermentation

A loopful of slant culture of *Flexibacter* sp. Q17897 (FERM 13305) was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of seed medium. The medium is composed of glucose 1.0%, tryptose 0.2%, yeast extract 0.1%, meat extract 0.1% (pH 7.0). The flask was shaken at 220 rpm on a rotary shaker for 3 days at 27°C. After incubation, an aliquot (4 ml) of seed culture was transferred to a 500 ml Erlenmeyer flask containing 100 ml of production medium having the following composition: polypepton 1.0%, dry yeast 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCl_2$ 0.038% (pH 7.2). Flasks were incubated at 27°C for 3 days at 220 rpm on a rotary shaker to afford 5 litters of culture broth.

Isolation

Culture broth (5 litters) was centrifuged at 5000 rpm for 15 minutes to remove the cells. The supernatant was adjusted to pH 7 with 0.1 N HCl and extracted twice with an equal volume of EtOAc. The EtOAc layers were combined, concentrated *in vacuo*, then subjected to silica gel column chromatography on Wakogel C-200 (Wako Pure Chemicals) with $CHCl_3$ - MeOH stepwise gradient (100 : 0 to 80 : 20). The active fractions eluted with $CHCl_3$ - MeOH (95 : 5) were combined and finally purified by HPLC on STR-ODS-H (10 i.d. × 250 mm; GL Science) with 35% MeCN (flow rate 4 ml/minute; UV detection at 215 nm) to afford YM-47141 (17.3 mg) and YM-47142 (7.9 mg), respectively.

Determination of HLE and Other Protease Inhibitory Activities

HLE inhibitory activity was determined by the method of SCHIESSLER *et al.*[8] with modifications. HLE (3.3 μg) dissolved in 33 μl of 0.001 N HCl and different amounts of inhibitor dissolved in 10 μl of MeOH were mixed with 624 μl of 0.2 M triethylamine-HCl buffer, pH 7.8. The mixture was incubated at 25°C for 10 minutes. The reaction was initiated by the addition of 333 μl of substrate solution (1.35 mg/ml of succinyl-Ala-Ala-Ala-*p*-nitroanilide in the same buffer mentioned above). The reaction mixture was incubated for 20 minutes before the addition of phenylmethyl sulfonyl fluoride. The hydrolysis of the substrate was monitored by the absorbance at 405 nm. Other protease inhibitory activities were determined by the method of MATSUOKA *et al.* (plasmin, thrombin, trypsin and kallikrein)[9], BARRETT (cathepsin G)[10], and DELMAR *et al.* (α-chymotrypsin)[11].

Results

Taxonomy of the Producing Organism

The producing organism, strain Q17897, was isolated from a soil sample collected in Zamami Island, Okinawa Prefecture, Japan. The morphological characteristics of strain Q17897 are summarized in Table 1. Strain Q17897 is a Gram-negative motile bacterium. The cells have a rod shape with a size of 0.2~0.4 × 0.4~15 μm. The color of the colony was orange. The physiological characteristics of strain Q17897 are summarized in Table 2. The growth temperature range was from 15~37°C. Strain Q17897 gave positive results in tests for ornithine decarboxylase and catalase. Acid formation was observed from D-glucose, lactose and maltose. The predominant isoprenoid quinone type was MK-7. The guanidine-plus-cytosine (G+C) content of the DNA was 47.5 mol%. Based on the taxonomic properties described above, strain Q17897 belongs to the genus *Flexibacter*[1,2].

Both morphological and physiological characteristics of strain Q17897 were compared with those of other *Flexibacter* species, *F. flexilis*, *F. elegans*, *F. filiformis*. The results are summarized in Table 3. Strain Q17897 differs from all these species in starch utilization as well as catalase and/or oxidase activities. Strain Q17897 is identified as *Flexibacter* sp. Q17897. This strain has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the access No. FERM 13305.

Table 1. Morphological characteristics of strain Q17897.

Gram strain	−
Motility	+
Color of coloney	Orange
Spore formation	−
Cell shape	Rod
Cell size	0.2~0.4 × 0.4~15 μm

Table 2. Physiological characteristics of strain Q17897.

VP test oxidase	−	O-F test	O type
Production of indole	−	Growth in 2% NaCl	+
H_2S	−	3% NaCl	−
pigment	+	Growth temperature	15∼37°C
Simmons citrate	−	Optimum temperature	27∼33°C
Utility of $NaNO_3$	−	Growth pH	5∼9
Gelatin liquefaction	+	Optimum pH	7∼8
Coagulation of milk	−	Anarobic growth	+
Peptonization of milk	+	G+C content of DNA	47.5 mol%
Ornithine decarboxylase	+	Quinone type	MK-7
Lysine decarboxylase	−	Acid formation from	
Oxidase	−	D-xylose	−
Catalase	+	D-glucose	+
Urease	−	Lactose	+
DNase	−	Maltose	+

Table 3. Taxonomic characteristics of strain Q17897 and other strains of Flexibacter.

	Q17897	*F. flexilis*	*F. elegans*	*F. filiformis*
Cell length	0.8∼>50	10∼>50	1.0∼>80	
Cell width	0.4∼0.8	0.5	0.4	0.6∼0.4
Color of colony	Orange	Orange	Bright orange	Golden yellow
Carbon utilization				
gelatin	+	+	+	+
starch	+	+	−	−
Production of indole	−	−	−	−
Production of H_2S	−	+	−	−
Nitrate reduction	−	−	−	−
Catalase	+	−	−	−
Oxidase	−	+	+	+
NaCl torelance (%)	2∼3	2.4	0.3	2.0
Temperature torelance (°C)	37∼40	40∼45	40∼45	40∼45
Optimum pH	7	7	7	7
G+C content of DNA (mol%)	47	40∼43	48	46∼47

Table 4. Physico-chemical properties of YM-47141 and YM-47142.

	YM-47141	YM-47142
Molecular formula	$C_{46}H_{62}N_8O_{13}$	$C_{43}H_{64}N_8O_{13}$
Molecular weight	934	900
HRFAB-MS (*m/z*)		
$(MH+H_2O)^+$		
Found:	953.4646	919.4825
Calcd:	953.4621	919.4777
UV	End absorption	End absorption
IR ν_{max} (KBr) cm^{-1}	3400, 2970, 1660, 1530	3300, 2960, 1650, 1530
$[\alpha]_D^{25}$ (*c*)	−10.1° (0.2)	−1.8° (0.5)

UV spectra and optical rotations were measured in MeCN.

Isolation and Physico-chemical Properties of YM-47141 and 47142

EtOAc extract of culture supernatant (5 litters) was subjected to silica gel column chromatography followed by HPLC to afford YM-47141 (17.3 mg) and YM-47142 (7.9 mg), respectively.

Physico-chemical properties of YM-47141 and YM-47142 are summarized in the Table 4. The IR spectra of YM-47141 and YM-47142 showed characteristic absorptions attributed to ester group (1660 cm^{-1}) and amide groups (1530 cm^{-1}). Amino acid analysis of 6 N HCl hydrolysate of YM-47141 and YM-47142 revealed the presence of Ala, Asp, Thr, Leu, and Phe (1 : 1 : 2 : 1 : 1). Both gave negative color reaction to ninhydrin indicating that the N-terminal of these peptide compounds is protected or cyclized. The details of the structure elucidation studies of YM-47141 and YM-47142 will be described in the succeeding paper[5].

Biological Properties of YM-47141 and 47142

YM-47141 and 47142 are potent inhibitors of elastase. The IC_{50} of YM-47141 and 47142 against HLE are 1.5×10^{-7} M and 3.0×10^{-7} M, respectively. Table 5 shows the inhibition activity of YM-47141 against several serine proteases. Although YM-47141 inhibits human leukocyte cathepsin G and bovine pancreatic α-

Table 5. IC_{50} of YM-47141 against various serine proteases.

Enzyme	Source	Substrate[a]	IC_{50} (M)
Elastase	Human leukocyte	Suc-AAA-pNA	1.5×10^{-7}
Plasmin	Human plasma	S-2251	$>10^{-4}$
Thrombin	Bovine plasma	S-2238	$>10^{-4}$
Trypsin	Bovine pancreas	S-2222	$>10^{-4}$
Kallikrein	Human plasma	S-2302	$>10^{-4}$
Cathepsin G	Hauman leukocyte	Suc-AAPF-pNA	2.3×10^{-6}
α-chymotrypsin	Bovine pancreas	Suc-AAPF-pNA	1.1×10^{-6}

[a] Substrates used: Suc-AAA-pNA, succinyl-alanyl-alanyl-alanine *p*-nitroanilide; S-2251, D-valyl-leucyl-lysine *p*-nitroanilide; S-2238, D-phenylalanyl-pipecolyl-arginine *p*-nitroanilide; S-2222, benzyl-isoleucyl-glutamyl*-glycyl-arginine *p*-nitroanilide; S-2302, D-prolyl-phenylalanyl-arginine *p*-nitroalanide; Suc-AAPF-pNA, succinyl-alanyl-alanyl-phenylalanyl-arginine *p*-nitroanilide.

* Mixture of free acid and methyl ester (1 : 1).

chymotrypsin, YM-47141 is most active against to HLE. Therefore, YM-47141 is a potent and fairly selective inhibitor of HLE.

Discussion

Production of proteinase inhibitors by microorganisms is well known[12]. Many serine and cysteine proteinase inhibitors possess C-terminal aldehyde group[13~15]. The aldehyde group is essential for the inhibitory activity due to the formation of a hemiacetal or hemithioacetal adduct with the nucleophilic hydroxy or thiol group of the serine and cysteine proteases[16]. In postostatin[17] the α-ketocarbonyl group is supposed to be the functional site for its inhibitory action[18]. Judging from the easy formation of the hydrates in solution, the inhibitory activities of YM-47141 and YM-47142 are most likely due to the formation of hemiketal between the enzyme active site serine and 2-carbonyl group of 2,3-dioxo-4-amino-6-methyl-heptanoic acid in the inhibitors. Recently, proteinase inhibitors with tricarbonyl structure have been synthesized[19]. YM-47141 and 47142 are the first example of proteinase inhibitors with tricarbonyl moiety being isolated from microbial sources.

References

1) Starkey, P. M. & A. J. Barrett: Human lysosomal elastase: Catalytic and immunological properties. Biochem. J. 155: 265~271, 1976
2) Janoff, A.: Elastase and emphysema. Current assessment of the protease-antiprotease hypothesis. Am. Rev. Respir. Dis. 132: 417~433, 1985
3) Merritt, T. A.; C. G. Cochrane, K. Holcomb, B. Bohl, M. Hallman, D. Strayer, D. Edwards & L. Gluck: Elastase and α_1 proteinase inhibitor activity in tracheal aspirates during respiratory distress syndrome. J. Clin. Invest. 72: 656~666, 1983
4) Janoff, A.: *In* Neutral proteases of human polymorphonuclear leukocytes, *Eds.*, K. Havermann & A. Janoff, Urban and Schwartzenberg, Baltimore: pp. 390~470, 1978
5) Orita, M.; K. Yasumuro, K. Kokubo, M. Shimizu, K. Abe, T. Tokunaga & H. Kaniwa: YM-47141 and 47142, new elastase inhibitors produced by Flexibacter sp. Q17897. II. Structure elucidation. J. Antibiotics 48: 1430~1434, 1995
6) Bergey's Mannual of Determinative Bacteriology 8th Edition, *Eds.*, R. E. Buchanan & N. E. Gibbons, The Williams & Wilkins Co., Baltimore, U.S.A., 1975
7) Bergey's Mannual of Systematic Bacteriology 9th Edition, *Eds.*, N. R. Krieg & J. G. Holt, The Williams & Wilkins Co., Baltimore, U.S.A., 1984
8) Schiessler, H.; K. Ohlsson, I. Olsson, M. Arnhold, Y. Birk & H. Fritz: Elastases from human and canine granulocytes, II. Interaction with protease with protease inhibitors of animal, plant, and microbial origine. Hoppe-Seyler's Z. Physiol. Chem. 358: 53~58, 1977
9) Matsuoka, S.; M. Futagami, H. Ohno, K. Imaki, T. Okegawa & A. Kawasaki: Inhibitory effects of ONO-3307 on various proteases and tissue thromboplastin in vitro and on experimental thrombosis in vivo. Japan J. Pharmacol. 51: 455~463, 1989
10) Barrett, A. J.: Method in enzymology Vol. 80. *Ed.*, L. Lorand, pp. 561~565, Academic Press, New York, 1981
11) Delmar, E. G.; C. Largman, J. W. Brodrick & M. C. Keokas: A sensitive new substrate for chymotrypsin. Analytical Biochemistry 99: 316~320, 1979
12) Umezawa, H.; Low-molecular-weight enzyme inhibitors of microbial origin. Annual Review of Microbiology 36: 75~99, 1982
13) Kondo, S.; K. Kawamura, J. Iwanaga, M. Hamada, T. Aoyagi, K. Maeda, T. Takeuchi & H. Umezawa: Isolation and characterization of leupeptins produced by Actinomycetes. Chem. Pharm. Bull. 17: 1896~1901, 1969
14) Umezawa, H.; T. Aoyagi, A. Okura, H. Morishima, T. Takeuchi & Y. Okami: Elastatinal, a new elastase inhibitor produced by actinomycetes. J. Antibiotics 26: 787~789, 1973
15) Umezawa, H.; T. Aoyagi, H. Morishima, S. Kunimoto, M. Matsuzaki, H. Hamada & T. Takeuchi: Chymostatin, a new chymotrypsin inhivitor produced by actinomycetes. J. Antibiotics 23: 425~427, 1979
16) Tomkinson, N. P.; I. J. Galpin & R. J. Beynon: Synthetic analogues of chymostatin. Inhibition of chymotrypsin and *Streptomyces griseus* proteinase A. Biochem. J. 286: 475~480, 1992

17) AOYAGI, T.; M. NAGAI, K. OGAWA, F. KOJIMA, M. OKADA, T. IKEDA, M. HAMADA & T. TAKEUCHI: Poststatin, a new inhibitor of prolyl endopeptidase, produced by *Streptomyces viridochromogenes* MH534-30F3. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. J. Antibiotics 44: 949~955, 1991

18) NAGAI, M.; K. OGAWA, Y. MURAOKA, H. NAGANAWA, T. AOYAGI & T. TAKEUCHI: Poststatin, a new inhibitor of prolyl endopeptidase, produced by *Streptomyces viridochromogenes* MH534-30-F3. II. Structure determination and inhibitory activities. J. Antibiotics 44: 956~961, 1991

19) FLYNN, G. A. & P. BEY (Merrell Dow Pharmaceuticals, Inc.): Novel peptidase and isomerase inhibitors. EP. 417721 A2, Mar. 20, 1991