FR901451†, A NOVEL INHIBITOR OF HUMAN LEUKOCYTE ELASTASE FROM *Flexibacter* sp.

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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A novel human leukocyte elastase (HLE) inhibitor, FR901451 was discovered in the fermentation broth of a bacteria. The bacteria was identified as *Flexibacter* sp. No. 758. FR901451 has a molecular weight of 1269 and a molecular formula of $C_{60}H_{79}N_{13}O_{18}$. The mode of inhibition against HLE is competitive, with a *Ki* value of 9.8×10^{-9} M.

Leukocyte elastases have been thought to be involved in the pathogenesis of many diseases such as pulmonary emphysema[1,2], rheumatoid arthritis[3], adult respiratory distress syndrome[4] and other inflammatory state[5,6]. In our search for microbially produced HLE inhibitors, we have discovered FR901451 as a product of *Flexibacter* sp. No. 758. This paper deals with the identification and fermentation of the producing microorganism, as well as isolation, physico-chemical and biological properties of FR901451.

Materials and Methods

Microorganism

Strain No. 758 was isolated from a soil sample obtained from Chiba prefecture, Japan. A lyophilized sample of this strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the accession No. FERM BP-3420.

Taxonomic Characterization

The methods described in BERGEY's Manual of Systematic Bacteriology (Vol. 3)[7] were employed principally for this taxonomic study. Morphological observation of the strain No. 758 was carried out by a light microscope and a scanning electron microscope with the cells grown on nutrient agar for 20 hours at 30°C. DNA was isolated by the phenol method of SAITO and MIURA[8]. The guanine-plus-cytosine (G+C) content of the DNA was determined by HPLC[9,10]. Isoprenoid quinone type was also analized by HPLC[11].

Fermentation

A seed medium (120 ml) containing yeast extract 0.5%, polypeptone 1.0% and NaCl 0.5% prepared

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in tap water was added to each of three 500-ml Erlenmyer flasks and was sterilized at 120°C for 30 minutes. A loopful of strain No. 758 from a mature slant, grown on bouillon agar at 30°C for 3 days, was inoculated into each of the seed flasks. The inoculated flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) at 30°C for 3 days. The resultant seed culture was inoculated into 20 liters of sterile fermentation medium. The medium consisted of 4.8% soluble starch, 1.6% corn steep liquor, 0.16% $(NH_4)_2SO_4$, 0.0096% $MgSO_4$ and 0.32% $CaCO_3$, prepared in tap water contained in a 30-liter stainless steel jar-fermentor. The pH of the medium was adjusted to 7.0 with NaOH prior to sterilization at 120°C for 30 minutes. The fermentation was carried out at 30°C for 2 days employing aeration at 20 liters/minute and stirring at 180 rpm.

The production of FR901451 in the fermentation was monitored by HPLC using a reverse phase column (YMC AM301 ODS 100 × 4.6 mm i.d., Yamamura Chemical Institute). The solvent system was methanol - acetonitrile - 364 mM $NH_4H_2PO_4$ (8 : 1 : 11, v/v), and the detection wave length was 210 nm.

Isolation

A hundred liters of acetone was added to the fermentation broth (100 liters) with stirring. The mixture was allowed to stand for 1 hour, and was filtered. The extract was concentrated under reduced pressure to remove acetone and was adsorbed onto polymeric adsorbent Diaion HP-20 (Mitsubishi Chemical Industrial Limited, 3 liters), washed with water and eluted with methanol. The eluate was concentrated and the resulted aqueous concentrate was adjusted to pH 2.0 with HCl, and washed with ethyl acetate. The water layer was separated and was adjusted to pH 7.0 with NaOH, and extracted with *n*-butanol. The butanol extract was concentrated to dryness and was applied to a column chromatography on silica gel (Kiesel gel 60, Merck, 450 ml). The column was washed with isopropanol, 90% aqueous (aq.) isopropanol and desired compound was eluted from the column with 1.5 liter of 80% aq. isopropanol. The eluate was concentrated and applied to a reverse phase silica, YMC-ODS-AM 120-S50 (Yamamura Chemical Institute, 300 ml), prepacked with 50% aq. methanol and developed with the same solvent. Next, the active eluate from the column was concentrated to dryness and dissolved in *n*-butanol - ethanol - water (4 : 1 : 1). The solution was subjected to a column chromatography on silica gel (85 ml) and developed with the same solvent. The fractions containing active substance were combined and concentrated under reduced pressure to give 37 mg of FR901451 as a white powder. The purity of isolated FR901451 was assessed by silica gel TLC using chloroform - methanol - NH_4OH (15 : 11 : 5) as a solvent and reverse phase HPLC. FR901451 showed a single spot at Rf 0.60 on a silica gel plate by spraying cerium sulfate. In reverse phase HPLC, the purity of FR901451 showed in excess of 97% by UV detection at 210 nm (column: YMC AM301 ODS 100 × 4.6 mm i.d.; mobil phase: methanol - acetonitril - 364 mM $NH_4H_2PO_4$ (8 : 1 : 11); flow rate: 1.0 ml/minute).

Physico-chemical Properties

Melting points were taken on a Yanagimoto micro melting point apparatus (uncorrected) and IR spectra were measured on a Nicolet 710 FT-IR spectrometer. Optical rotation was determined on a Jasco DIP-140 polarimeter using a 10 cm-microcell. ^1H NMR spectra (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured with a Bruker AMX500 spectrometer. The chemical shifts are reported in ppm relative to internal sodium 3-trimethylsilyl-propionate-d_4 and coupling constants are expressed in Hz. Low-resolution FAB-MS spectra were obtained on a VG ZAB-SE mass spectrometer and amino acid analysis was performed on a HITACHI 835 amino acid analyzer.

Protease Inhibition Assay

The method was described previously[12]. Briefly, 25 μl of 2 mM methoxysuccinyl-$(Ala)_2$-Pro-Val-*p*-nitroanilide (substrate) and 50 μl of inhibitor or vehicle were mixed in wells of 96 well-microtiter plate. The absorbance of the mixture at 415 nm was measured using a microplate reader. After the measurement, 25 μl of a solution of human sputum elastase (HSE, 6 μg/ml) was added and incubated for 30 minutes at room temperature. Then the absorbance at 415 nm was measured and percent inhibition was calculated. The effects of inhibitors for other proteases were assayed similarly using 2 mM *N*-succinyl-$(Ala)_3$-*p*-nitroanilide as substrate for porcine pancreatic elastase (PPE, Type IV, 5 μg/ml final), 2 mM *N*-α-benzoyl-Arg-*p*-nitroanilide as substrate for bovine pancreatic trypsin (Type I, 16 μg/ml final) and 2 mM methoxysuccinyl-$(Ala)_2$-Pro-Met-*p*-nitroanilide as substrate for bovine pancreatic chymotrypsin (Type II,

1.5 μg/ml final). HSE was obtained from Elastin Products Company Inc., U.S.A. and was used as HLE. All substrates, proteases and α1-AT (from human plasma) were purchased from the Sigma Chemical Co.

Results

Taxonomic Characterization of the Strain No. 758

1) Morphological characteristics

Strain No. 758 was a Gram-negative and non-sporulating bacterium. The strain was slender rods or sometimes filaments, motile by gliding. Colonies of the strain were semi-transparent, orange yellow, circular, convex and entire-edged on nutrient agar. The strain No. 758 was 0.8~1.0×2.0~18 μm in size without flagella (Fig. 1). These results were shown in Table 1.

2) Physiological characteristics

Physiological characteristics of the strain No. 758 were shown in Tables 2, 3. The growth temperature was from 17°C to 44°C. The strain No. 758 was oxidase positive, catalase positive and flexirubin reaction positive. Nitrate was reduced to nitrite. This strain hydrolized gelatin, casein and esculin. Starch hydrolysis was negative. Decomposing ability of cellulose, chitin, agar and alginate was negative. The

Fig. 1. Scanning electron micrograph of *Flexibacter* sp. No. 758. Bar = 5 μm.

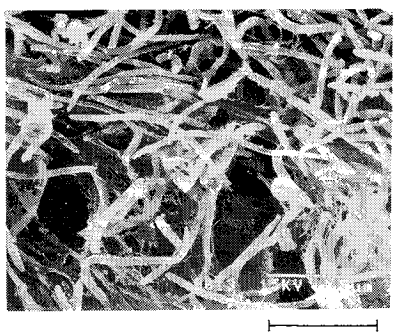


Table 1. Morphological characteristics of strain No. 758.

Gram stain	Negative
Color of colony	Orange yellow
Cell shape	Slender rods
Cell size	0.8~1.0×2.0~18 μm
Motility	Gliding
Flagella	Negative
Spore formation	Negative

Table 2. Physiological characteristics of strain No. 758.

Conditions	Characteristics
Growth temperature	17~44°C
Growth in air	Positive
Growth on MacConkey agar	Negative
Growth on marine agar (DIFCO marine agar 2216)	Positive
Flexirubin reaction	Positive
Catalase	Positive (weak)
Oxidase	Positive
Tolerance to NaCl	0~2%
H_2S (SIM)	Negative
Simmons citrate	Negative
Nitrare reduction	Positive
Indole	Negative
Gelatin liquefaction	Positive
Casein hydrolysis	Positive (weak)
Esculin hydrolysis	Positive
Starch hydrolysis	Negative
Tween 80 hydrolysis	Negative
Degradation of	
Colloidal chitin	Negative
Carboxymethylcellulose	Negative
Agar	Negative
Alginate	Negative
ONPG test	Negative
DNase	Positive
Lysine decarboxylase	Negative
Arginine dihydrolase	Positive (weak)
Ornitine decarboxylase	Negative
G+C content of DNA	49.8 mol%
Isoprenoid quinone	MK-7

Table 3. Acid formation from sugars and utilization of sugars by strain No. 758.

	Acid	Growth
D-glucose	Positive	Positive
D-xylose	Positive	Positive
D-fructose	Negative	Negative
D-galactose	Positive	Positive
D-mannitol	Negative	Negative
Sucrose	Positive	Positive
Lactose	Positive	Positive
Maltose	Positive	Positive
Salicine	Negative	Negative

G+C content of DNA was 49.8 mol%. Major isoprenoid quinone was MK-7. Acid formation was observed from D-glucose, D-xylose, D-galactose, sucrose, lactose and maltose. The following compounds were utilized as a sole carbon source: namely, D-glucose, D-xylose, D-galactose, sucrose, lactose and maltose.

3) Identification

According to BERGEY's Manual of Systematic Bacteriology (Vol. 3), the strain No. 758 was considered to belong to the genus *Flexibacter* from those characteristics described above. We therefore identified this strain as *Flexibacter* sp. No. 758.

Physico-chemical Properties

The physico-chemical properties of FR901451 are summerized in Table 4. FR901451 is soluble in methanol and water, slightly soluble in acetone, and insoluble in ethyl acetate. Its color reaction were positive to cerium sulfate, Ninhydrin and Ehrlich reagents, but negative to Molisch reagent. The molecular formula was established to be $C_{60}H_{79}N_{13}O_{18}$ by elemental analysis and FAB-MS. The ^1H NMR and ^{13}C NMR spectra are shown in Figs. 2 and 3. Amino acid analysis of a 6 N HCl hydrolysate of FR901451 revealed the presence of Asp, Thr, Ser, Pro, Leu, Phe, Lys and Trp (3:2:1:1:1:1:1:trace). The even amino acids, identified above, account for all the sequence of the amino acids present in **I** (Scheme 1). The molecular formula of I shows 28 degrees of unsaturation. However, the above fragments lead to 25 degrees of unsaturation. The remaining three degrees unsaturation were then due to the cyclic nature of the compound. The details of the structure elucidation studies of FR901451 is described else-

Table 4. Physico-chemical properties of FR901451.

Appearance	White powder
MP	243~245°C (dec.)
$[\alpha]_D^{23}$	−15° (*c* 0.65, H_2O)
Elemental analysis	Calcd for $C_{60}H_{79}N_{13}O_{18}\cdot 10H_2O$: C 49.68, H 6.88, N 12.55
Found:	C 49.95, H 6.28, N 12.42
FAB-MS (*m/z*)	1270 $(M+H)^+$
UV $[\lambda]_{max}^{MeOH}$ nm (ε)	290 (3,891), 281 (4,512), 275 (4,259)
IR (KBr)	3390, 3070, 2970, 2880, 1740, 1660, 1530, 1450, 1410, 1380, 1350, 1250, 1190, 1110, 1080, 1010, 750, 700, 670, 660, 620, 600 cm^{-1}
Amino acid Analysis (Molar)	Asp (3), Thr (2), Ser (1), Pro (1), Leu (1), Phe (1), Lys (1), Trp (trace)

Fig. 2. ^1H NMR spectrum of FR901451 (500 MHz).
(H_2O-D_2O-CD_3COOD, 9:1:1).

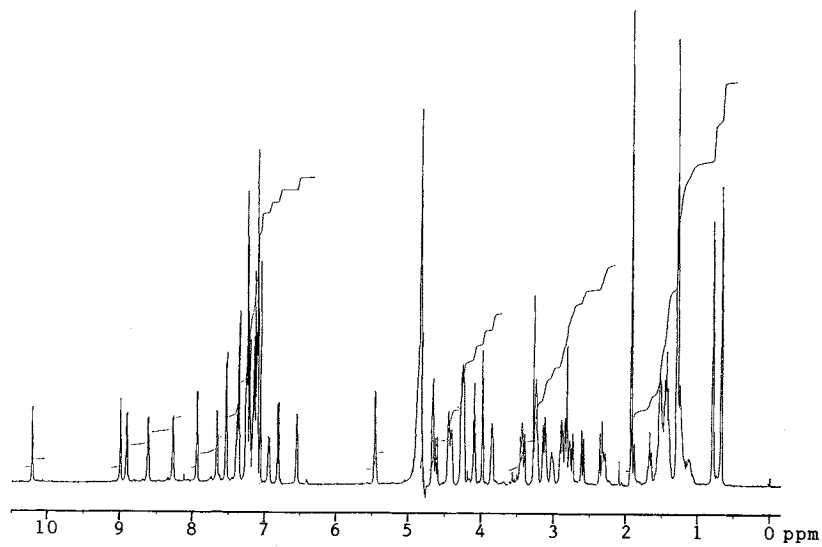
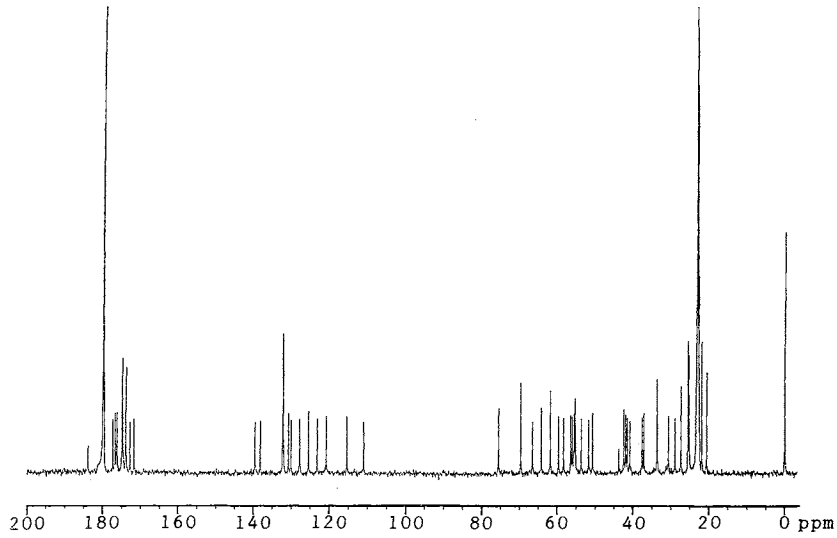
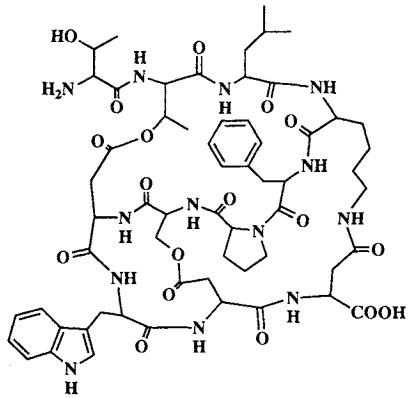


Fig. 3. ^{13}C NMR spectrum of FR901451 (125 MHz).
(H_2O-D_2O-CD_3COOD, 9:1:1).



Scheme 1.



FR901451 (I)

where[13].

Biological Properties

Effects of FR901451 and α1-AT on Various Proteinases

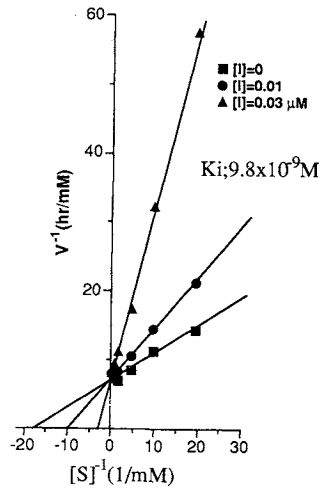
Inhibitory effects of FR901451 and α1-AT against HLE, PPE, chymotrypsin and trypsin were determined. As shown in Table 5, FR901451 showed inhibition against HLE, PPE and chymotrypsin with IC_{50}s of 2.3×10^{-7}, 2.7×10^{-7} and 1.1×10^{-7} M, respectively. However, FR901451 was a very

Table 5. IC_{50} values of inhibitors for various proteinases.

Inhibitor (M)	Proteinase			
	HLE	PPE	Chymotrypsin	Trypsin
FR901451	2.3×10^{-7}	2.7×10^{-7}	1.1×10^{-7}	7.9×10^{-5}
α1-AT	4.4×10^{-8}	1.6×10^{-7}	5.8×10^{-8}	5.7×10^{-7}

α1-AT; α1-antitrypsin.

Fig. 4. Lineweaver-Burk plot of inhibition of HLE by FR901451.



weak inhibitor of trypsin. α1-AT, an endogenous inhibitor (MW; 54 kDa) of serine protease, inhibited all enzymes tested with IC_{50}s of lower than 10^{-6} M.

Mode of Inhibition of FR901451

Lineweaver-Burk analysis[14] showed that the inhibition against HLE by FR901451 was competitive with substrate (Fig. 4). The *Ki* value was 9.8×10^{-9} M.

Acute Toxicity

The 50% lethal dose of FR901451 by intraperitoneal injection to male *ddY* mice (6-week-old) was more than 1 g/kg.

Discussion

Previously, we have discovered FR901277 in the fermentation broth of *Streptomyces resistomycificus* No. 7622[12]. Further screening for HLE inhibitors of bacterial products resulted in the discovery of FR901451 as a product of *Flexibacter* sp. No. 758.

FR901451 is a potent inhibitor of HLE with low toxicity. Therefore FR901451 may be useful in modurating the destructive process associated with chronic inflammatory diseases such as emphysema. The effect of FR901451 on animal models of pulmonary emphysema will be described in succeeding paper[15].

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