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Characterization of an Elastase Inhibitor Produced by *Streptomyces lavendulae* SMF11

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Abstract An elastase inhibitor, SMFEI02, was isolated from culture broth of *Streptomyces lavendulae* SMF11. The inhibitor was purified by ultrafiltration followed by XAD-7 column and Dowex-1 anion-exchange chromatographies, and preparative HPLC. The molecular formula was determined to be $C_{14}H_{16}N_2O_2$ (MW 244) by HRFAB-MS analysis. The inhibitor was identified to be a diketopiperazine *cyclo*(*S*-Phe-*S*-Pro) by the optical rotation value and NMR spectral data, and showed inhibitory activities for trypsin, chymotrypsin, cathepsin B, and papain as well as elastase with the K_i values ranging from 1.78 mM to 2.86 μM. The inhibition showed a competitive mode for elastase, chymotrypsin, and cathepsin B, whereas it showed a noncompetitive mode for trypsin and papain.

Key words: Elastase, inhibitor, diketopiperazine, *Streptomyces lavendulae*, competitive inhibition

Elastase is defined by its ability to release soluble peptides from insoluble elastin fibers by a proteolytic process called elastinolysis [3], and is mostly found in the pancreas, leukocytes and macrophages. Elastase found in pancreas or leukocytes belongs to the chymotrypsin family of serine proteinases. Pancreatic elastase (EC 3.4.21.36, PE) can hydrolyze soluble proteins such as casein, hemoglobin, α_1-antichymotrypsin [11], and connective proteoglycans as well as elastin [18], and the activity of PE is responsible for the destruction of elastic tissue of intrapancreatic blood vessels in the course of acute hemorrhagic pancreatitis [6]. The major physiological function of leukocyte elastase (EC 3.4.21.37, LE) is probably to digest bacteria and immune complexes phagocytosed by the polymorphonuclear leukocytes, and human LE is thought to play a pathological role in lung emphysema [7], cytic fibrosis, the adult respiratory distress syndrome (ARDS), rheumatoid arthritis, and infectious diseases. Macrophage elastase (EC 3.4.24.65), classified as a member of the matrix metalloprotease subfamily [2], may cause tissue destruction if it is excessively or inappropriately expressed. It is postulated that elastase is appropriately expressed during the host inflammatory response to remodel extracellular matrix and enhances macrophage recruitment.

The indications of involvement of elastase in all these diseases have prompted researchers to screen elastase inhibitors as possible therapeutic agents. Naturally occurring or engineered proteinaceous inhibitors such as plasma-derived α_1-proteinase inhibitor and recombinant mucus proteinase inhibitors are known to have medicinal effects [4]. However, these proteinaceous inhibitors showed inhibitory activities for various plasma proteases, and putative instability in the biological systems. To obtain a selective and stable inhibitor, low molecular natural products have been screened. Elastatinal [16] and elasnin [12, 13] were previously identified from microorganisms and SMFEI02 is reported in this work.

SMFEI02, *cyclo*(*S*-Phe-*S*-Pro), is known to be produced by fungi such as *Alternaria alternata* [15] and *Rosellinia necatrix* [5], having weak phytotoxic activity. The compound was also detected in the cultures of *Streptomyces rochei* 87051-3, *Aspergillus flavipes*, and *Candida albicans*, but its role was not identified. Thus, this is the first report on *cyclo*(*S*-Phe-*S*-Pro), with protease inhibitory activity, isolated from the culture broth of *S. lavendulae* SMF11. This paper describes the purification procedures, physicochemical properties, structure elucidation of SMFEI02, and also the inhibition profile for various proteases.

Materials and Method

Microorganism

Streptomyces lavendulae SMF11 isolated from soil was used [8]. Stock cultures of the organism were

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maintained on ISP Medium 4 agar plates and transferred every month.

Media and Culture Conditions

A loopful of strain from the plate was inoculated into five 1-l baffled-flasks containing 300 ml of sterile seed medium consisting of 3% glucose, 2.2% soybean meal, 0.3% peptone, and 0.4% $CaCO_3$, and incubated on a rotary shaker at 30°C for 2 days at 150 rpm. The resultant seed culture was inoculated (10%, v/v) into a 50-l jar fermentor (Korea Fermentor Co., Korea) containing 15 l of sterile fermentative seed medium consisting of 3% glucose, 2.2% soybean meal, 0.3% peptone, 0.04% KH_2PO_4, and 0.16% K_2HPO_4. The fermentation was carried out at 30°C for 2 days employing aeration at 0.5 vvm and stirring at 300 rpm. For the production of inhibitor SMFEI02, 15 l of seed culture was transferred to a 300-l fermentor (Korea Fermentor Co., Korea) containing 150 l of medium consisting of 4% glucose, 2% peptone, 0.01% KH_2PO_4, 0.04% K_2HPO_4, 0.0005% $FeSO_4 \cdot 7H_2O$, 0.001% $ZnSO_4 \cdot 7H_2O$, and 0.0075% $MgSO_4 \cdot 7H_2O$. The fermentation was carried out under the same condition as for the seed culture, and the culture pH was maintained at 7.0 by the addition of either 1 N HCl or 1 N NaOH automatically.

Instrumental Analysis

The inhibition kinetics were carried out on a UV-160 (Shimadzu, Japan) UV/Visible spectrophotometer. Mass spectra were taken on a JMS AX505WA (Jeol, Japan) spectrometer (Inter-University Center for Natural Science Research Facilities, Seoul, Korea). Optical rotation was taken with a J720 (Jasco, Japan) polarimeter with a 5-cm micro cell. UV spectrum was taken on a UV-160 (Shimadzu, Japan) UV/VIS spectrophotometer. ^{1}H-NMR, ^{1}H-^{1}H-COSY, ^{13}C-NMR, and DEPT spectra were recorded with a UNITY 300 (Varian, U.S.A.) spectroscope using CD_3OD as solvent. Silica TLC plates (Silica gel 60 F_{254}) were purchased from Merck Company (Darmstadt, Germany).

Analytical Methods

Biomass was determined as dried mycelium weight after drying at 80°C for 24 h. The concentration of glucose was estimated by the dinitrosalicylic acid method [10]. The concentration of ammonium ion was analyzed by a specific ion analyzer (Model EA940, Orion Research, U.S.A.). The elastase inhibitory activity was assayed as follows. A 100 µl of elastase solution (100 µg/ml) was incubated for 5 min at 37°C in parallel with 100 µl of distilled water as a control or with inhibitor in 550 µl of 0.1 M Tris-HCl buffer at pH 8.0 in a spectrophotometer cuvette. The inhibitor was prepared as filtrate by ultrafiltration of culture broth using Microcon 3000 (Amicon, U.S.A.). The reaction was started by the addition of 250 µl of Suc-Ala-Ala-Ala-*p*NA (400 µg/ml) as a substrate and the enzyme activity was estimated by measuring the amount of *p*-nitroanilides liberated from the substrate using E_{405}=9,620 $mol^{-1}cm^{-1}$. The inhibitory activity was calculated from the formula: % Inhibition=100×(A-B)/A, where A is enzyme activity without the inhibitor and B is enzyme activity with the inhibitor. One unit of the inhibitory activity was defined as the amount of the inhibitor needed for 50% inhibition of elastase.

Kinetic Measurements of the Inhibitory Activity against Proteases

For the studies of inhibition kinetics, the initial velocities of amidase activity of various proteases including elastase (porcine pancreas), chymotrypsin (bovine pancreas), trypsin (bovine pancreas), cathepsin B (bovine spleen), and papain (papaya latex) were determined by continuous spectrophotometric assay using the absorption coefficient of *p*-nitroaniline, E_{400}=10,500 $mol^{-1}cm^{-1}$. Each protease was added to a concentration of 0.01-0.3 µM dissolved in buffers: 0.1 M Tris-HCl buffer (pH 7.5) for elastase, chymotrypsin and trypsin and 0.1 M sodium phosphate buffer containing 1.33 mM EDTA (pH 6.0) for cathepsin B (with 2 mM cysteine) or papain (with 5 mM cysteine). The inhibitors were preincubated with the enzyme for 10 min and the reactions were then started by the addition of a substrate to a final concentration of 0.1-0.5 mM. The K_i value for each protease was obtained from Lineweaver-Burk plots [9].

RESULTS AND DISCUSSION

Production of Inhibitor

Mycelium growth and production of the elastase inhibitor in a jar fermentor were monitored, and the resultant data are shown in Fig. 1. Mycelium growth was accompanied by a somewhat delayed production of the elastase inhibitor. The maximal activity of the elastase inhibitor was observed after 40 h of cultivation.

Isolation and Purification of the Inhibitor SMFEI02

The procedure used for the isolation of inhibitor SMFEI02 is shown in Fig. 2. The culture broth (150 l) was centrifuged at 9,200 rpm by continuous centrifugation (Westfalia, Germany) to remove mycelia. The resulting supernatant was filtered through microfiltration membranes (Altenburger, Germany) of 0.5 and 0.1 µm pore size and the filtrate was further filtered using ultrafiltration membranes (Millipore Co., U.S.A.) of NMWL 3,000 Da. The filtrate was concentrated using a centrifugal thin film vacuum evaporator (Okawara MFG Co. Ltd., Japan) at 13 torr and 64°C. The concentrate was adsorbed onto Amberlite XAD-7 resin. The column was washed with 15% methanol and the active substance was eluted with 80% methanol. The

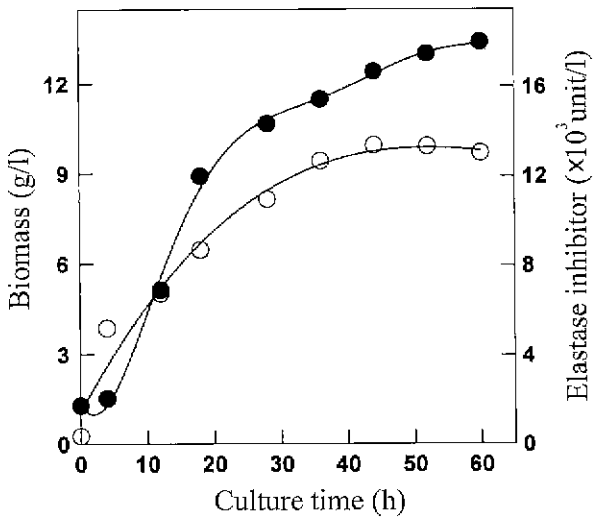

Fig. 1. Batch culture data for the production of elastase inhibitor from *Streptomyces lavendulae* SMF11.
The cultures were performed at 30°C with a working volume of 150-l, stirred at 300 rpm, and the pH was maintained to 7.0. Aeration was maintained at a rate of 0.5 vvm. Change of biomass (●) and elastase inhibitor (○) were measured

active fraction was concentrated and then applied to a column of Dowex-1 (Cl^- form). The inhibitor passed through the column without binding. The fraction was concentrated to dryness to give a yellowish syrup. Preparative HPLC (JAI, Japan) was carried out with a Hypersil H5ODS column (10×250 mm, Hichrom, U.K.). The active substance was eluted with a linear gradient of 24 to 30% acetonitrile in 20 mM sodium phosphate buffer (pH 6.0) at a flow rate of 2.5 ml/min. The fraction was concentrated and fractionated by a μBondapak CN column

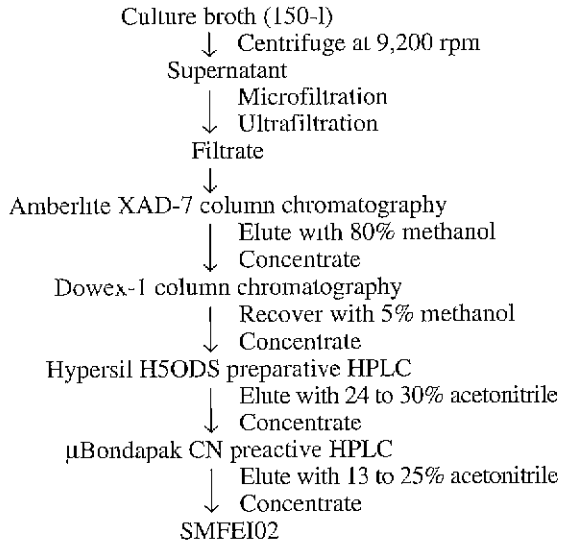

Fig. 2. Isolation procedure of the elastase inhibitor SMFEI02 from the culture broth of *Streptomyces lavendulae* SMF11.

Table 1. Physicochemical properties of the SMFEI02.

Appearance of crystal	White solid
Molecular formula	$C_{14}H_{16}N_2O_2$
HRFAB-MS (m/z)	
Found $(M+H)^+$	245.1295
Calcd $(M+H)^+$	245.1290
$[\alpha]_D^{25}$ (*c* 0.7, MeOH)	-72°
R_f value	0.69[a]
	0.55[b]
Solubility	
soluble in	Acetone, ethylacetate, methanol, H_2O
slightly soluble in	Ether

[a,b]SMFEI02 was developed on silica gel TLC plates with *n*-butanol:acetic acid:H_2O=4:1.2 and *n*-butanol:H_2O=9:1, respectively.

(7.8×300 mm, Waters) with a linear gradient of 13 to 25% acetonitrile at a flow rate of 2.5 ml/min. The active fraction yielded white powder of pure active compound.

Physicochemical Properties and Determination of Chemical Structure

Physicochemical properties of the SMFEI02 are summarized in Table 1. The SMFEI02 was soluble in acetone, ethylacetate, methanol, and water, and slightly soluble in ether. The molecular weight and formula of the SMFEI02 were determined to be 244 Da and $C_{14}H_{16}N_2O_2$, respectively, by the HRFAB-MS analysis. The ultraviolet absorption spectrum of the SMFEI02 dissolved in methanol showed two maximum absorption peaks at 221.4 nm and 258.0 nm, which indicated the presence of a phenyl group(s) (Fig. 3). The ^1H- and ^{13}C-NMR assignments of the SMFEI02 are listed in Table 2. The ^1H-NMR spectrum revealed that the SMFEI02 contained an aromatic ring, four methylenes, and two methines. The ^{13}C-NMR spectrum and a DEPT experiment showed five aromatic methines, two other methines, four methylenes, and three non-protonated carbons. ^1H-^1H COSY experiment established the presence of the coupling of a methine proton at 4.04 ppm (H-8) to methylene protons at 1.21 and 2.07 ppm (H-7a and H-7b), and the couplings among H-5, H-6, and H-7. These 1D and 2D NMR spectral data demonstrated that the SMFEI02

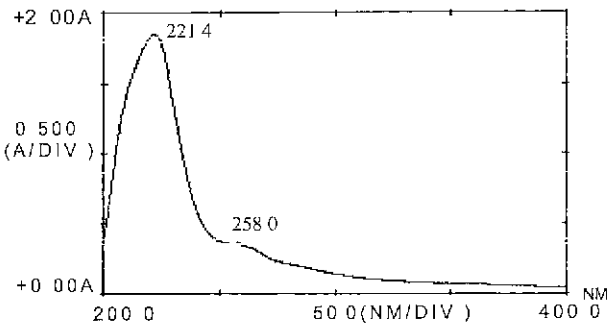

Fig. 3. Ultraviolet spectrum of the SMFEI02 in methanol.

was *cyclo*(phenylalanylprolyl). Its absolute stereochemistry was determined on the basis of the optical rotational value and chemical shift of α-CH of proline. The optical rotation $[\alpha]_D$ of the SMFEI02 at 25°C was -72° (*c* 0.7 in MeOH), suggesting that proline has an *S*-configuration [1]. As shown in Table 2, the proline H_α appeared at δ4.04, which is in good agreement with the value of δ4.05 of *cyclo*(*S*-Phe-*S*-Pro) in literature [19], whereas it is known that *cyclo*(*R*-Phe-*S*-Pro) has the value of δ2.85. Therefore, these data suggested that the phenylalanine has an *S*-configuration.

Thus, the SMFEI02 is a diketopiperazine with the structure of (3*S*,8a*S*)-hexahydro-3-(phenylmethyl)pyrrolo [1.2a]pyrazine-1,4-dione (Fig. 4). The compound was previously isolated from the cultured broth of *Rosellinia necatrix* Berlese and shown to have retardative activity for the growth of plant seedling and plant roots [5]. It was also isolated from liquid cultures of *Alternaria alternata*, the causal agent of black leaf blight of spotted knapweed (*Centaurea maculosa* Lam), and shown to have weak phytotoxic activity [15]. The mechanism of action of a *cyclo*(*S*-Phe-*S*-Pro) has not been characterized. The fact that *cyclo*(*S*-Phe-*S*-Pro) had the inhibitory activity against proteases shown in Table 3 provided a possibility of its phytotoxicity by interacting with cellular enzyme(s).

Table 2. ^1H and ^{13}C NMR data for the SMFEI02.

Position No.	^1H (ppm)[a]	^{13}C (ppm)[b]
1	-	166.85
3	4.20 (t, *J*=4.8)	57.64
4	-	170.88
5	3.34 (m)	45.93
	3.51 (dt, *J*=12.0, 8.4)	
6	1.77 (m)	22.75
7	1.21 (m)	29.34
	2.07 (m)	
8	4.04 (dd, *J*=10.5, 6.3)	60.03
9	3.14 (d, *J*=4.8)	38.13
1'	-	137.35
2'	7.1-7.3 (m)	131.00
3'	7.1-7.3 (m)	129.42
4'	7.1-7.3 (m)	128.03

Chemical shifts and coupling constants are expressed in ppm and Hz, respectively.
[a]300 MHz in CD_3OD.
[b]75 MHz in CD_3OD.

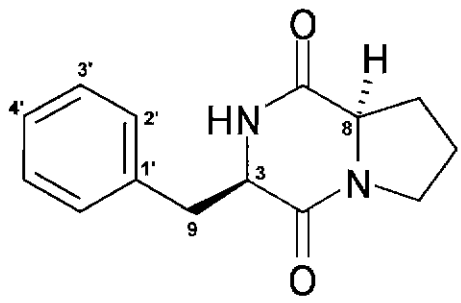

Fig. 4. Structure of the SMFEI02.

Characters of the SMFEI02

The inhibitory effects of the SMFEI02 against various proteases as well as elastase are shown in Table 3. The SMFEI02 had a K_i of 1.78 mM towards elastase, indicating

Table 3. Inhibition of various proteases by the SMFEI02.

Protease	Substrate	K_i(M)
Serine protease		
Elastase	Suc-$(Ala)_3$-*p*NA	1.78×10^{-3}
Chymotrypsin	Suc-$(Ala)_2$-Pro-Phe-*p*NA	2.50×10^{-5}
Trypsin	Z-Phe-Arg-*p*NA	3.50×10^{-5}
Cysteine protease		
Cathepsin B	Z-Arg-Arg-*p*NA	9.13×10^{-6}
Papain	Z-Phe-Arg-*p*NA	2.86×10^{-6}

Suc, succinyl; *p*NA, *p*-nitroanilide; Z, benzyloxycarbonyl.

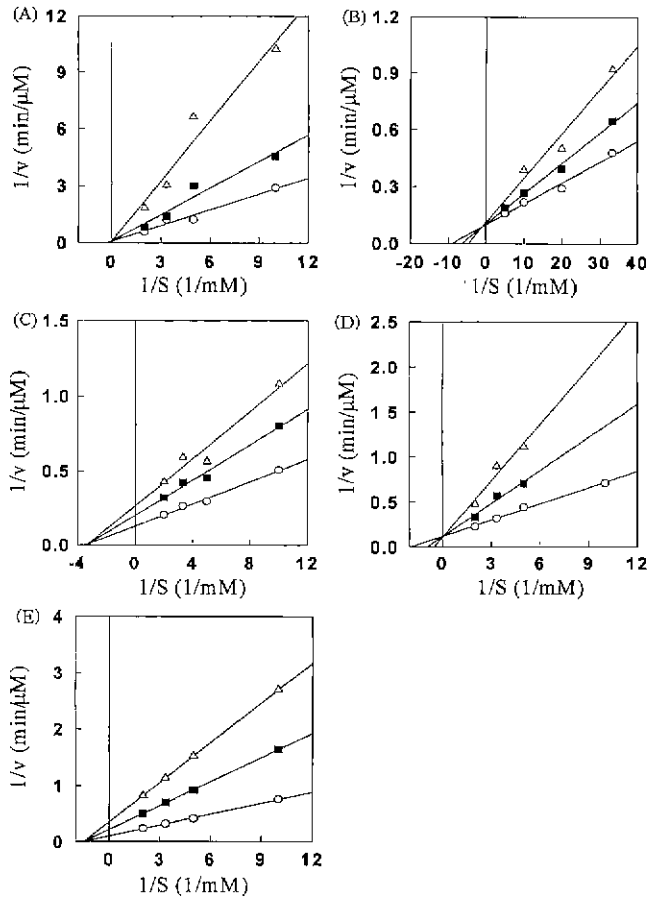

Fig. 5. Inhibition of proteases by the SMFEI02.
Lineweaver-Burk plot of reciprocal substrate concentration against reciprocal rate of hydrolysis by elastase (A), chymotrypsin (B), trypsin (C), cathepsin B (D), and papain (E) with (■, △) and without (○) the SMFEI02.

that it is less potent than elastatinal (K_i=0.24 μM) [17] and elasnin (K_i=70 μM) [14] (Table 3). Towards chymotrypsin, trypsin, cathepsin B, and papain, the compound had K_i's ranging from 3 to 35 μM and showed competitive or noncompetitive inhibition, as determined by Lineweaver-Burk plots (Fig. 5). The above-described results showed that SMFE102 was a nonspecific inhibitor for cysteine proteases as three related serine proteases, and might provide a new insight for designing inhibitors of elastase and other proteases as a lead compound.

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