ISOLATION AND CHARACTERIZATION OF ELASNIN, A NEW HUMAN GRANULOCYTE ELASTASE INHIBITOR PRODUCED BY A STRAIN OF *STREPTOMYCES*

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Elasnin, a new human granulocyte elastase inhibitor, produced by the strain of KM-2753 designated as *Streptomyces noboritoensis* KM-2753 has been isolated from the fermentation broth by column chromatography on silica gel and neutral alumina. Elasnin is a neutral, colorless, and viscous oil ($n_D^{17}=1.4983$, $[\alpha]_D^{18}$ $-0.9°$, λ_{max}^{EtOH} 291 nm (ε, 7,760)) having a molecular formula of $C_{24}H_{40}O_4$ (MW 392) as shown by its elemental analysis and mass spectrum. Elasnin markedly inhibits human granulocyte elastase, but it is almost inactive against pancreatic elastase, chymotrypsin, and trypsin. At 1.3 μg/ml (3.3×10^{-6} M), elasnin is 50% inhibitory to human elastase, but it causes 50% inhibition of pancreatic elastase at 30.1 μg/ml (76.8×10^{-6} M).

In the course of our screening program for useful inhibitors against human granulocyte elastase, a new elastase inhibitor "elasnin" was found in the culture filtrates of a strain of *Streptomyces*[1]. The present paper deals with taxonomic studies of the producing strain, fermentation, isolation, and characterization of elasnin. This culture also produced anti-candida activity.

Materials and Methods

Taxonomic studies

The elasnin producing strain KM-2753 was isolated from a soil sample collected at Horinouchi-cho, Kitauonuma-gun, Niigata Prefecture in Japan. The strain KM-2753 was cultivated on various media described by WAKSMAN[2] and ISP (International Streptomyces Project)[3] at 27°C or 37°C and growth, aerial mycelia and soluble pigments were observed after a period of 14 days. The culture was also grown and observed on gelatin medium at 22°C. The utilization of carbon sources was tested in PRIDHAM and GOTTLIEB's liquid media containing 1% of various carbon sources[4]. The color and hue numbers indicated were based on those of the Color Harmony Mannual (4th edition) published by Container Cooperation of America.

Methods of culture

Different media were employed for growth and maintenance of the culture. Seed medium (pH 7) contained dextrin, glucose, soybean meal, yeast extract, and $CaCO_3$ (20, 2, 15, 3 and 3 g respectively per liter of tap water). Production medium (pH 7) contained glucose, soybean meal, and NaCl (20, 20 and 1 g respectively per liter of tap water). The stock culture of strain KM-2753 was maintained at room temperature (25°C) as an agar slant (WAKSMAN's medium), with transfers made every few weeks. Culture stored for long periods of time were generally kept at 4°C or as freeze-dried stock.

A loopful from a 7-day agar culture of strain KM-2753 was inoculated into 100 ml of seed medium in a 500-ml SAKAGUCHI flask and incubated for two days at 27°C. This growth was used to inoculate 30-liter jar-fermentors containing 20 liters of the production medium. Fermentation was for

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4 days at 27°C under the following conditions: temperature, 27°C; aeration, 10 liters/min.; agitation, 250 r.p.m.; and pressure, 0.5 kg/cm^2.

Enzymes

Human granulocyte elastase was prepared from fresh whole blood as described by FEINSTEIN *et al.*[5]: human granulocyte elastase extract was prepared by homogenizing granulocyte cells in 0.35 M sucrose, 5 mM Tris buffer, pH 7.5 and sodium heparin (500 u/ml) with a Teflon pestle homogenizer. The homogenate was centrifuged at 16,000×*g* for 20 minutes. The pellet containing the granules was suspended in 50 mM Tris buffer, pH 7.5 containing 1 M NaCl and subjected to 6 cycles of freeze-thawing. The lysed granules were extracted by stirring the mixture at 4°C for 2 hours. The supernatant obtained after centrifugation at 16,000×*g* for 20 minutes contained elastase activity.

Chymotrypsin was obtained from Sigma Chemicals Co., trypsin (1: 250) from Difco Co., U.S.A. and hog pancreatic elastase from Miles Laboratories, U.S.A. The elastase substrate, N-*t*-Boc-Ala-Ala-Pro-Ala-*p*-nitroanilide (Boc-AAPAN) was a gift from Dr. M. ZIMMERMAN, Merck Sharp & Dohme, U.S.A. The chymotrypsin substrate, N-benzoyl-L-tyrosine ethyl ester (BTEE) was purchased from Nakarai Chemicals Ltd., Japan, and the trypsin substrate, N-benzoyl-arginine-*p*-nitroanilide (BAPA) from Seikagaku Kogyo Co., Ltd., Japan. The four tetrapeptide chloromethyl ketones, Ac-$(Ala)_2$-Ala-AlaCH_2Cl, Ac-$(Ala)_2$-Pro-AlaCH_2Cl, Ac$(Ala)_2$-Pro-IleCH_2Cl and Ac-$(Ala)_2$-Pro-Val-CH_2Cl were gifts from Dr. J. C. POWERS of the Georgia Institute of Technology, U.S.A., and elastatinal was a gift from Dr. T. AOYAGI, Institute of Microbial Chemistry, Tokyo.

Assay for inhibitory activity

Both pancreatic and granulocyte elastases were assayed at 30°C using a solution containing 0.4 mM Boc-AAPAN as substrate with 0.05 M Tris-HCl buffer (pH 7.5) and in the presence of 10% dimethylsulfoxide (DMSO). Activity was determined by measuring the production of *p*-nitroanilide using a Shimadzu UV-210A spectrophotometer set at 410 nm. The concentration of both enzymes corresponded to an extinction change of E_{410}=0.03/min in 1.6 ml of assay mixture. Chymotrypsin[6] and trypsin[7] were assayed similarly using 1.07 mM BTEE and 2.2 mM BAPA as substrates, respectively.

DMSO solutions of elasnin and other inhibitors previously prepared were added to each substrate solution at specified concentrations prior to the additions of the enzymes. The residual enzymatic activities were compared with those of controls in the absence of inhibitors. One unit was defined as the concentration of elasnin or other inhibitors that inhibited 50% of the enzyme activity.

Results and Discussion

Taxonomic Characteristics of Strain KM-2753

Morphological characteristics

The morphology of the strain cultured on inorganic salts-starch agar for 14 days at 27°C was microscopically observed (Fig. 1). The aerial mycelium of the strain grew well in both synthetic and organic agar media. Thin upright strands of aerial hyphae developed. The spores were usually oval form, although some spores had constrictions in the inner walls that suggested partial fissure to equally sized units.

Cultural and physiological characteristics

Cultural and physiological characteristics of strain KM-2753 are listed in Tables 1 and 2, respectively. The utilization of carbon sources by the strain is shown in Table 3. The cultural and physiological characteristics may be summarized as follows: aerial mycelium is relatively long and straight with no characteristic appearance; aerial mycelium is white to grayish white; soluble pigment is light yellow to yellowish white on various media; and it produces melanoid pigment.

Notwithstanding the differences indicated in Table 4, it seems that the new isolate closely resemble *Streptomyces noboritoensis* ISONO, YAMASHITA, TOMIYAMA, SUZUKI and SAKAI (1957)[8].

Fig. 1. Electronmicrograph of the spores of *Streptomyces noboritoensis* KM–2753 (Inorganic salts-starch agar 14 days, ×12,000)

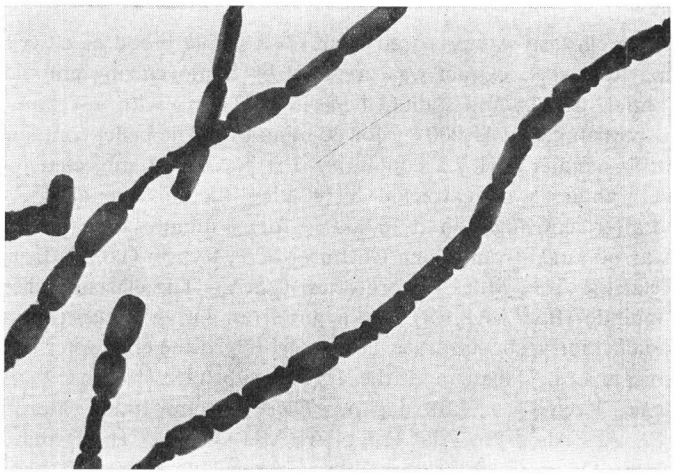


Table 1. Cultural characteristics of strain KM–2753

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Glucose-nitrate agar	thin and moderate, deep brown (4pl)	deep brown (4pl)	no or little formation	light yellow
Sucrose-nitrate agar	no or little formation	—	—	—
Glycerol-calcium malate agar	raised without crease, abundantly grown, chestnut brown (4ni)	chocolate (4nl)	no or little formation	cork tan (4ie)
Glucose-asparagine agar	more or less raised with creases, abundantly grown, cork tan (4ie)	cork tan (4ie)	no or little formation	light brown (4ng)
Glycerol-asparagine agar	more or less raised, no crease, abundantly grown, maple (4le)	maple (4le)	no or little formation	dark luggage tan (4pg)
Inorganic salts-starch agar	more or less dispersed, no crease, abundantly grown dark brown (3pn)	dark brown (3pn)	cotton-like, moderately grown, white	citron (1gc)
Tyrosine-agar	moderately grown, dark brown (3nl)	dark brown (3pn)	white	dark brown (4pn)
Yeast-malt agar	raised with creases, abundantly grown, topaz (3ne)	topaz (3ne)	thinly grown, ashes (5fe)	yellow maple (3ng)
Oatmeal agar	more or less dispersed, abundantly grown, yellow maple (3ng)	yellow maple (3ng)	no or little formation	yellow maple to dark brown (3ng to 3pn)
Peptone-yeast-iron agar	not raised, moderately grown, covert tan (2ge)	covert tan (2ge)	no or little formation	light brown (31g)
Glucose-peptone agar	more or less raised, abundantly grown, oak brown (4pi)	oak brown (4pi)	formed partly as white spots	light brown (4ng)
Nutrient agar	more or less raised with creases, moderately grown, dark brown (4pn)	dark brown (4pn)	moderately formed, moss-like, natural (2dc)	deep brown (4pl)

Consequently, the strain KM–2753 has been classified as a *Streptomyces noboritoensis* and designated as *Streptomyces noboritoensis* KM–2753 with an accesion number of FERM–P 4024.

Isolation and Purification of Elasnin

A typical time course of elasnin production by the strain KM–2753 is shown in Fig. 2. To isolate elasnin, the broth filtrate (10 ml) was extracted with an equal volume of ethyl acetate. The extract (3 ml) was evaporated and the residue was dissolved in 100 μl DMSO and 10 μl samples were used for assays. The elasnin assay was described in the text. The anti-candida substance was assayed microbiologically using *Candida albicans* as a test organism. Residual glucose was estimated by using the ortodin method[9].

The concentration of elasnin at 97 hours was about 75 u/ml and it decreased almost linearly beyond this time. The production of the anti-candida substance started about 24 hours after inoculation and its maximum concentration was attained after 72 hours and then decreased gradually.

Table 2. Physiological properties of strain KM–2753

Melanin formation	+
Tyrosinase reaction	+
H_2S production	+
Nitrate reduction	+
Liquefaction of gelatin	+
Hydrolysis of starch	+
Coagulation of milk	+
Peptonization of milk	+
Cellulolytic activity	−
Temp. range for growth	15~37°C

Table 3. Utilization of carbon sources by strain KM–2753

Response	Carbon source
Positive	D-Glucose, L-rhamnose, D-mannitol
Probably positive	L-Arabinose, raffinose
Negative	Sucrose, D-fructose, D-xylose, *i*-inositol

Fig. 2. A typical time course of elasnin production by strain KM–2753

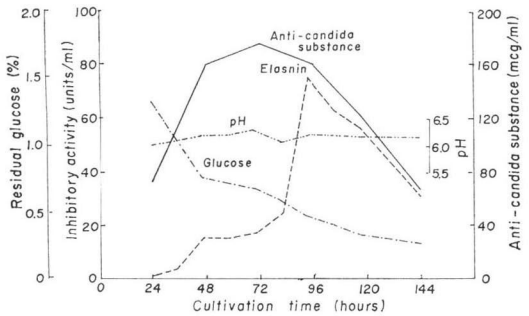


Table 4. Differences of *S. noboritoensis* and strain KM–2753

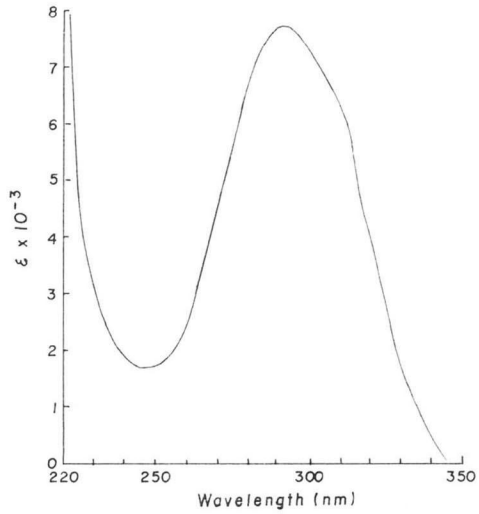
	S. noboritoensis	Strain KM–2753
Utilization of rhamnose	−	+
Liquefaction of gelatin	± or −	+
Hydrolysis of starch	±	+
Elasnin production	±	+

Fig. 3. Isolation of elasnin from the culture broth of strain KM–2753

	Yield (g)	Sp. act. (u/mg)
Whole broth (70 liters)		
↓ extracted with ethyl acetate, concentrated *in vacuo*		
Crude oil	100	56
↓ adjusted to pH 10 with 0.5 N NaOH, re-extracted with benzene, concentrated *in vacuo*, chromatographed on silica gel 60, eluted with benzene, benzene – acetone (40: 1)		
Dark red oil	12	120
↓ chromatographed on neutral alumina eluted with benzene – EtOAc (5: 1), EtOAc		
Brown oil	9	152
↓ chromatographed on silica gel treated with acetate buffer, pH 4.8, eluted with benzene – EtOAc (20: 1)		
Yellow oil	6.4	280
↓ chromatographed on silica gel (Mallinckrodt Inc. Silic AR CC–4), eluted with benzene – acetone (200: 1)		
Pale yellow oil	2.8	360
↓ chromatographed on preparative silica gel plate, developed with benzene – ethanol (40: 1 and 10: 1)		
Colorless oil	1.4	420

Culture broths (70 liters) of strain KM-2753 obtained at 96 hours from 100-liter fermentors were used for large scale isolation of elasnin. The isolation procedure is shown in Fig. 3. The ethyl acetate extract from whole broth was evaporated *in vacuo* and the residue was adjusted to pH 10 with 0.5 N NaOH solution to form benzene-insoluble salts of the fatty acids and anti-candida substance. The anti-candida activity was identified as belonging to the antimycin group by UV and IR spectra, and biological activities. The benzene extract was evaporated to dryness. The residual oily preparation was serially chromatographed on silica gel, neutral alumina (for decolorizing), silica gel treated with acetate buffer (pH 4.8) and silic AR CC-4 silica gel. In further purification by preparative TLC, the oil was added to preparative silica gel thin-layer plates (20×20 cm, thickness 0.5 mm) and development was with benzene – ethanol (10: 1). The spot corresponding to elasnin (Rf 0.5, detected under UV light (2537 Å) and with I_2) was collected and eluted with benzene – acetone (5: 1) to obtain an oily product.

Fig. 4. UV spectrum of elasnin (in EtOH)



Characteristics of Elasnin

Properties of elasnin are as follows: $n_D^{17}=1.4983$; $[\alpha]_D^{18}$ −0.9° (*c* 1.0, EtOH); and UV absorption maximum at 291 nm (ε, 7,760 in EtOH) as depicted in Fig. 4.

The molecular formula was determined as $C_{24}H_{40}O_4$ (M^+ *m/e* 392) by its elemental analysis (Anal. Found: C, 73.42%; H, 10.34%, O, 16.24%; Calcd. for $C_{24}H_{40}O_4$: C, 73.47%; H, 10.20%, O, 16.33%) and mass spectrum (Fig. 5). The infrared spectrum (Fig. 6) indicated absorptions due to hydroxyl(s) at 3430 cm^{-1}, methyl or methylene(s) at 2860 and 2960 cm^{-1}, carbonyl group(s) at 1715 and 1665 cm^{-1}, and double bond(s) at 1636 cm^{-1}. The ^{13}C-NMR spectrum indicated the presence of a ketone carbonyl carbon (δ 206.9), carbonyl and olefinic carbons (δ 104.3 ~ 165.5), a methine (δ 54.7), and methylene (δ 40.2). Further, characteristic signals due to methylene and methyl carbon were observed at δ 22.6 ~ 31.9, and δ 13.9, respectively as shown in Fig. 7.

Fig. 5. Mass spectrum of elasnin

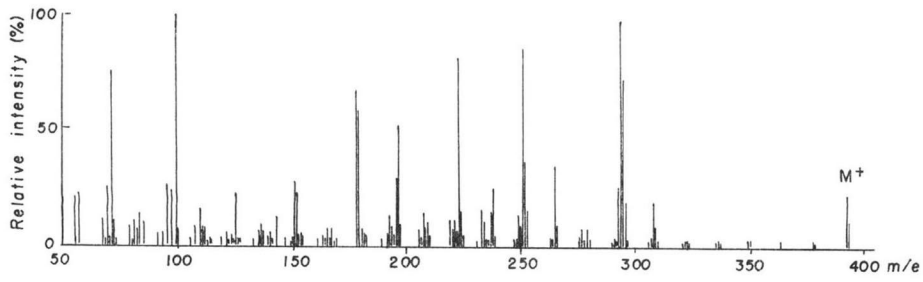


Fig. 6. IR spectrum of elasnin (CCl_4)

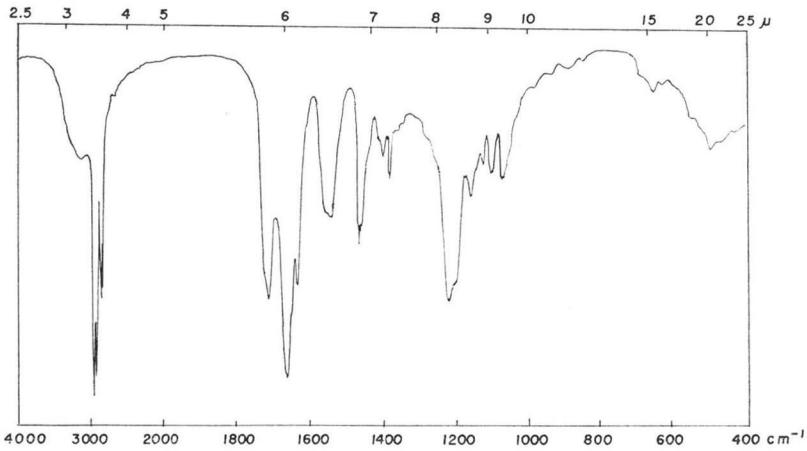
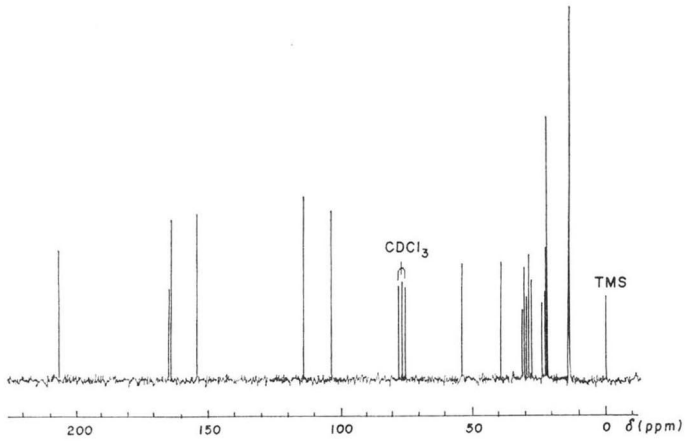


Fig. 7. ^{13}C-NMR spectrum of elasnin ($CDCl_3$)



Elasnin gave positive tests with $KMnO_4$, $FeCl_2$, I_2 and BCG indicator, but negative BEILSTEIN, ninhydrin, MOLISCH and 20% H_2SO_4 tests. It was soluble in DMSO, methanol, ethanol, acetone, chloroform, benzene, ethyl acetate and hexane, but insoluble in water. Physicochemical properties are summarized in Table 5.

Inhibitory Activity of Several Inhibitors on Various Proteases

The inhibitory activities of several inhibitors against granulocyte and pancreatic elastases and other proteases are presented in Table 6. Elasnin exhibited 50% inhibition of granulocyte elastase at 1.3 μg/ml (3.3 μM), whereas 25 times higher concentrations of elasnin were required for 50% inhibition of pancreatic elastase. Chymotrypsin and trypsin were almost unaffected by elasnin. Our results with elastatinal, isolated from a species of actinomycetes by UMEZAWA *et al.*[10] also indicate that it is very inhibitory to porcine pancreatic elastase, but that it has low activity against human leukocyte elastase[11]. Four chloromethyl ketone analogs[12] were classified into two groups; one has potent

Table 5. Physicochemical properties of elasnin

Appearance	Colorless viscous oil
$[\alpha]_D^{18}$	$-0.9°$ (c 1, EtOH)
n_D^{17}	1.4983
Mass	M^+, m/e 392 Monoacetate; M^+, m/e 434 (434.2984. Calcd. for $C_{26}H_{42}O_5$, 434.3032)
Elemental analysis	C, 73.42%; H, 10.34%; O, 16.24%. (Calcd. for $C_{24}H_{40}O_4$, C, 73.47%; H, 10,20%; O, 16.33%)
UV (EtOH)	λ_{max} 291 nm (ε, 7,760)
IR (CCl_4)	3430, 2960, 2860, 1715, 1665, 1635, 1550, 1465, 1455, 1380, 1220, 1160, 1100, 1070 cm^{-1}
Color reaction	Positive: $KMnO_4$, $FeCl_2$, I_2, BCG Negative: BEILSTEIN, ninhydrin, MOLISCH, 20% H_2SO_4
Solubility	Soluble in MeOH, EtOH, acetone, benzene, EtOAc, $CHCl_3$ Insoluble in H_2O

Table 6. Inhibitory activity of several inhibitors on various proteases

Protease	ID_{50}* (mcg/ml)					
	Elasnin	Elastatinal	R–Ala–Ala–R′	R–Pro–Ala–R′	R–Pro–Ile–R′	R–Pro–Val–R′
Granulocyte elastase	1.3	>200	>200	>200	7.8	18
Pancreatic elastase	30.1	0.63	14	3.8	2.1	6.4
Chymotrypsin	82	>200	>200	>200	>200	>200
Trypsin	90	>200	>200	>200	>200	>200

* 50% Inhibitory dose: the concentration which inhibits 50% of the enzyme activity
R: –Ala–Ala–Ac
R′: $-CH_2Cl$

inhibitory activity against pancreatic elastase, and the other markedly inhibits both pancreatic and granulocyte elastases.

From these results, it can be concluded that elasnin is a specific inhibitor of human granulocyte elastase.

Elasnin has a low toxicity. The acute toxicity (LD_{50}) in mice (*ddy*-strain, 3 weeks of age) was 290 mg/kg and >1,000 mg/kg by intraperitoneal and oral administrations, respectively. Elasnin had no antibacterial or antifungal activity at a concentration of 1,000 μg/ml (400 u/ml of elasnin activity).

Elastatinal[10], several synthetic agents[13,14], and certain *cis*-unsaturated fatty acids[15] have previously been reported to be active against pancreatic or/and granulocyte elastases. Inhibitory activities of these inhibitors against various proteases and physicochemical properties are different from those of elasnin.

The specific action of elasnin is interesting in connection with its potential application to acute arthritis, various inflammations, pulmonary emphysema[16], and pancreatitis[17]. Elasnin may facilitate a better understanding of these disease processes.

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