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ELASNIN, A NEW HUMAN GRANULOCYTE ELASTASE INHIBITOR PRODUCED BY A STRAIN OF STREPTOMYCES

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Summary

Elasnin, a new human granulocyte elastase inhibitor, has been isolated from Streptomyces noboritoensis KM-2753. Elasnin is a neutral, lipophilic colorless and viscous oil (n_D^{17} =1.4983, $[\alpha]_D^{18}$ -0.9°, λ_{max}^{EtOH} 291 nm (ε, 7760)). The molecular formula was $C_{24}H_{40}O_4$ (M.W.: 392) as determined by its elemental analysis and mass spectrum. Elasnin inhibits markedly human granulocyte elastase, but is almost ineffective for pancreatic elastase, trypsin, chymotrypsin, thermolysin and papain.

Introduction

Inhibitors for trypsin, chymotrypsin, papain, thermolysin, and pepsin have recently been isolated from culture broth of microorganisms (1). Pancreatic elastase inhibitors have also been found in various sources such as plants (2), mammalian organs (3) and microorganisms (4). More recently, several synthetic agents (5-6) and certain cis-unsaturated fatty acids (7) as inhibitors of pancreatic and human granulocyte elastases have been reported. With respect to functional importance, human granulocyte elastase is currently suggested to be involved in pathological processes such as acute arthritis, various inflammations, pulmonary emphysema (8) and pancreatitis (9).

Our interest in the screening of human granulocyte elastase inhibitor also arises from the probable involvement of this enzyme in the diseases caused by tissue destruction. In the course of screening of human granulocyte elastase inhibitors from microorganisms, we found that the strain KM-2753 produces a new inhibitor having a potent anti-elastase activity. The present paper deals with the fermentation, isolation and properties of

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elasnin, the novel human granulocyte elastase inhibitor, produced by Streptomyces noboritoensis KM-2753.

Materials and Methods

Human granulocyte elastase was prepared from fresh whole blood according to the procedure of Feinstein and Janoff (10).

Chymotrypsin was obtained from Sigma Chemical Co., and other enzymes were generously offered by the following suppliers: trypsin (Difco), pancreatic elastase (Miles), thermolysin (Seikagaku Kogyo Co., LTD.) and papain (Wako Pure Chemical Industries, LTD.). N-t-Boc-Ala-Ala-Pro-Ala-p-nitroanilide (Boc-AAPAN) was a gift of Dr. M. Zimmerman, Merck Sharp & Dohme. N-benzoyl-L-tyrosine ethyl ester (BTEE) was purchased from Nakarai Chemicals LTD. and N-benzoylarginine-p-nitroanilide (BAPA) from Seikagaku Kogyo Co., LTD.

Both pancreatic and granulocyte elastases activities were assayed in a solution of 0.4 mM Boc-AAPAN as a substrate containing 0.05 M Tris-HCl buffer (pH 7.5) and 10% dimethylsulfoxide (DMSO) at 30°C by measuring the production of p-nitroanilide at 410 nm using a Shimadzu UV-210A spectrophotometer The concentration of both enzymes corresponded to an extinction change of E_{410}=0.03/min. in 1.6 ml assay mixture. Chymotrypsin and trypsin were assayed similarly using 1.07 mM BTEE and 2.2 mM BAPA as a substrate, respectively. Thermolysin and papain were assayed by the change of absorbance at 280 nm during the hydrolysis of 2% casein.

A DMSO solution of elasnin previously prepared was added to the substrate solution at specified concentration prior to the addition of the enzyme. The residual enzyme activity was compared to that of a control under the absence of inhibitor. One unit was defined as the concentration of elasnin which inhibits 50% of the enzyme activity.

The following media were employed for the production of elasnin. Seed medium contained 2% of dextrin, 0.2% of glucose, 1.5% of soybean meal, 0.3% of yeast extract, and 0.3% of $CaCO_3$, pH 7.0. Production medium contained 2% of glucose, 2% of soybean meal, and 0.1% of NaCl, pH 7.0.

The stock culture of the strain KM-2753 was maintained as an agar slant (Waksman's medium). A 7-day agar culture was inoculated into a seed medium (100 ml) in a Sakaguchi flask, incubated for two days at 27°C, and the used as a seed culture for production of elasnin. Fermentation was carried out using a 30-liter Jar fermentor containing 20 liters of the production medium for 4 days at 27°C.

Results and Discussion

The strain KM-2753 was designated as Streptomyces noboritoensis KM-2753 on the basis of its morphological observations, cultural characteristics and physiological properties (11).

Time course of the production of elasnin is shown in Fig. 1. The concentration of elasnin at 94 hrs was about 75 u/ml and almost linearly decreased beyond 94 hrs. While a broth at 96 hrs was employed as a starting material for the isolation of elasnin. The isolation procedure of elasnin

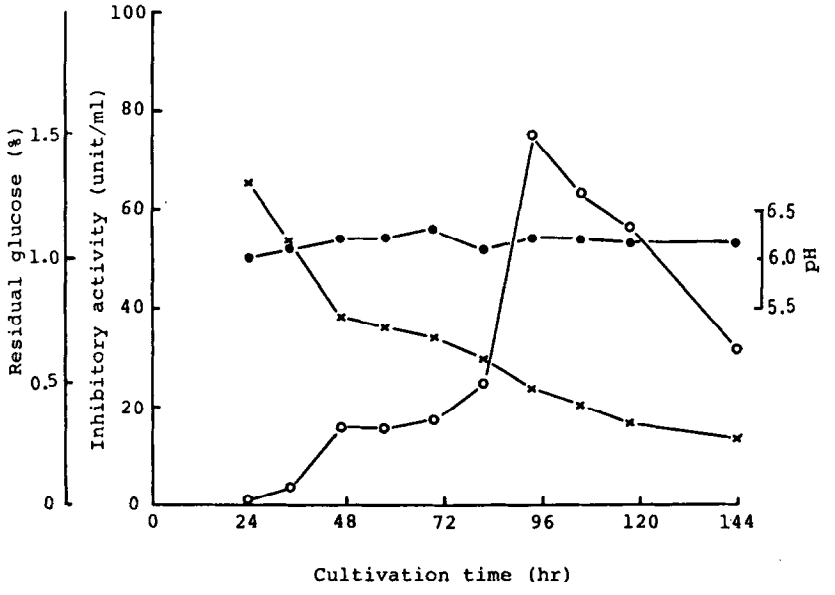

Figure 1. Time course of elasnin production by Streptomyces noboritoensis KM-2753. O—O, inhibitory activity; ×—×, residual glucose; ●—●, pH of broth. Cultivation was performed using a 30-liter jar fermentor containing 20 liters of the medium described in the text. Culture conditions were as follows: agitation, 300 rpm; temp., 27°C; aeration, 10 liters/minute. Elasnin activity was assayed by the method described in the text. Residual glucose was estimated by using ortodin method (12).

is shown in Fig. 2. The ethyl acetate extract from the whole broth were chromatographed on silica gel. Further purification was carried out by silica gel preparative thin layer chromatograph to obtain a pure substance. It showed a single spot having R_f values 0.65 with benzene-acetone (5:1), 0.45 with benzene-ethyl acetate (8:1) and 0.50 with benzene-ethanol (10:1) upon thin layer chromatography (Merck, Kieselgel GF_{254}) as detected both under UV light (2537 Å) and with I_2 vapor, and indicated only one peak by gas chromatography (Yanagimoto gas chromatograph Model 550-F).

Properties of elasnin were as follows: n_D^{17}=1.4983, $[\alpha]_D^{18}$ -0.9° (C 1.0, EtOH); and showed an absorption maximum at 291 nm (ε, 7760 in EtOH). The molecular formula was $C_{24}H_{40}O_4$ as shown by its elemental analysis. (C, 73.42%; H, 10.34%; O, 16.24%) and mass spectrum (M^+ 392). This was

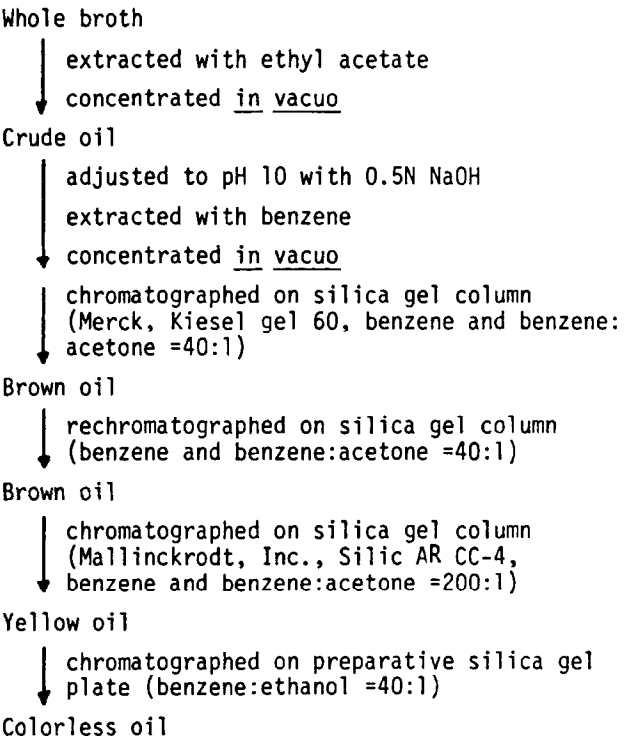

Figure 2. Purification procedure of elasnin

further confirmed by the mass spectrum of the monoacetate, M^+ m/e 434 (434.2984 calcd. for $C_{26}H_{42}O_5$, 434.3032). The infrared spectrum showed the absorption due to hydroxyl at 3430 cm^{-1}, methyl or methylene at 2860 and 2960 cm^{-1}, carbonyl group at 1715 and 1665 cm^{-1} and double bond at 1636 cm^{-1}. It was soluble in almost any organic solvent such as DMSO, methanol, ethanol, acetone, chloroform, benzene, ethyl acetate and n-hexane, but insoluble in water.

The activity of elasnin against pancreatic and granulocyte elastases, and the other proteases is shown in Table 1. As seen in Table 1, elasnin showed 50% inhibition of granulocyte elastase at 1.3 mcg/ml, whereas pancreatic elastase required 25 times higher concentration of elasnin for 50% inhibition. Chymotrypsin, trypsin, thermolysin, and papain were almost unaffected by elasnin.

Elasnin had a low toxicity and the acute toxicity (LD_{50}) in mice was

Table 1. Inhibitory activity of elasnin on various proteases.

Protease	ID_{50}* (mcg/ml)
Granulocyte elastase	1.3
Pancreatic elastase	30.1
Chymotrypsin	82
Trypsin	90
Thermolysin	>200
Papain	>200

*: 50% inhibitory dose- the concentration which inhibits 50% of the enzyme activity

290 mg/kg by intraperitoneal administration and >1000 mg/kg by orally. On the other hand, elastatinal isolated from a species of actinomycetes by Umezawa et al. (4) has a potent inhibitory activity against porcine pancreatic elastase, but shows very low activity against human leukocyte elastase (13). From these results, it can be concluded that elasnin is a specific inhibitor of human granulocyte elastase.

The specific action of elasnin is interesting in connection with its potential application to arthritis, inflammation, emphysema and pancreatitis. Elasnin will be able to clarify the mechanistic processes of these disease and to clearly demonstrate the differences between pancreatic and granulocyte elastases.

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