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Original Article

Isolation and Characterization of a Novel Elastase Inhibitor, AFLEI from *Aspergillus flavus*

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Abstract

A novel elastase inhibitor from *Aspergillus flavus* (AFLEI) was isolated, and biochemical properties of AFLEI were examined. Column chromatography using diethylaminoethyl (DE) 52-Cellulose and Sephadex G-75 was used to purify the inhibitor. The final preparation was found to be homogeneous as indicated by a single band after disc polyacrylamide gel (PAGE) and isoelectric focusing electrophoreses. AFLEI had a molecular weight of 7,525.8 as determined by TOF-MS (time of flight mass spectrometry). The elastolytic activity of elastases from *A. flavus, A. fumigatus* and human leukocytes were inhibited by AFLEI. However, this activity from porcine pancreas elastase, trypsin, chymotrypsin, thrombin, and Ac_1-Proteinase from snake venom was not affected by AFLEI. The fibrinogenase activity of the elastase from *A. flavus* was inhibited by AFLEI. AFLEI was inhibited by α_2-macroglobulin. However, ethylenediaminetetraacetic acid (EDTA-2Na), benzamidine, chymostatin, tosyl phenylalanine chloromethyl ketone (TPCK) and dithiothreitol (DTT) did not show any inhibitory effect on the elastase inhibitory activity of AFLEI.

Key words: *Aspergillus flavus*, elastase inhibitor, isolation, property

INTRODUCTION

Aspergillosis is a common mycosis in immunocompromised hosts undergoing chemotherapy. It is caused by inhalation of high spore concentrations of the *Aspergillus* species. It is reported that various proteases are produced from *A. fumigatus*[1-5], *A. flavus*[6, 7] and *A. niger*[8, 9]. Since 28% of lung tissue is composed of elastin, a strong relationship is suggested between elastase and the pathogenesis of aspergillosis.

From a practical standpoint, elastase-producing strains promote a higher death rate in immunodeficient mice than non-elastase producing strains. Elastase-producing strains destroy lung tissue[10, 11] and an elastase produced by *Aspergillus* has been confirmed as a definitive virulence factor of aspergillosis[12].

Frosco *et al.*[13] observed that the purified monoclonal antibody to elastase derived from *A. fumigatus* possesses the ability to inhibit the elastase produced by *A. fumigatus*. Ulinastatin, which contains an enzyme inhibitor, was administered by way of intravenous drip infusion concomitantly with antifungal agents to patients of pulmonary aspergillosis[14-18]. It is very likely that the elastase inhibitor is an effective therapy of aspergillosis.

It has been shown that *A. fumigatus* and *A. flavus* produced elastase inhibitors[19], although it had not previously been know that *Aspergillus* spp. produced this elastase inhibitor. A novel elastase inhibitor from *Aspergillus flavus* (AFLEI) is the first reported isolation of an elastase

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inhibitor from *A. flavus*. In this paper, we report the isolation, and biochemical properties of a novel elastase inhibitor from *A. flavus* (AFL-1 strain) from a patient.

MATERIALS AND METHODS

Strain and Reagents

Aspergillus flavus (AFL-1 strain) was identified and isolated from sputum from a patient with allergic bronchopulmonary aspergillosis. Elastases from *A. flavus* and *A. fumigatus* were purified by the method of Hasegawa *et al.*[2, 7]. Succinyl L-alanyl- L-alanyl- L-alanyl p-nitroanilide (STANA) was purchased from the Peptide Institute Inc. (Osaka, Japan). Yeast carbon base and casamino acids were obtained from Difco Lab., (Detroit, MI, USA). Elastin, elastase from porcine pancreas and human leukocytes, trypsin, chymotrypsin and N-1-naphtyletylenediamine dihydrochloride were from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Sodium nitrite, fibrinogen and dimethylsulfoxide (DMSO) were procured from Katayama Chemical Industry, Ltd. (Osaka). Ammonium sulfamate and collagen (Type I, II, III, IV) were supplied from Wako Pure Chemical Industries, Ltd. (Osaka). Membrane filters were purchased from Advantec Toyo Co., Ltd. (Tokyo, Japan). DE52-Cellulose was obtained from Whatman Biochemical (Clifton, NJ, USA). Sephadex G-75 was purchased from Pharmacia (Uppsala, Sweden). α_2-Macroglobulin was supplied from Boehringer Mannheim (Mannheim, Germany). Thrombin was obtained from Mochida Pharmaceutical Co., Ltd. (Tokyo). Proteinase from *S. epirermidis* was prepared in this laboratory. Other chemicals used were of analytical grade and purchased from commercial sources.

Production of elastase inhibitor

A sufficient number of microorganic spores were cultured in 100 m*l* synthetic medium of 0.1% yeast carbon base containing 1% casamino acid as a nitrogen source. The cultures were incubated for 7 days at 37°C without shaking. The culture broth was filtered aseptically through a 0.22 μm pore size membrane filter, and the cell-free culture supernatant was used as the source of crude inhibitor.

Assay for elastolytic activity

Elastolytic activity of the elastase was assayed by the method of diazocoupling measuring p-nitroanilide (p-NA) released from 50 mM STANA in DMSO, which was used as the substrate. To determine the extent of STANA digestion, 0.1 m*l* of the enzyme solution and 0.9 m*l* of 50 mM Tris-HCl buffer, pH 7.5, and 20 μl of 50 mM STANA solution were incubated for 60 min at 37°C. The reaction was stopped by adding 1 m*l* of 10% trichloroacetic acid (TCA). Then 0.2 m*l* of 0.1% sodium nitrite, 0.5% ammonium sulfamate and 0.1% N-1-naphtyletylenediamine dihydrochloride were added to the reaction solution and the color developed was read at 550 nm. One unit of STANA hydrolase activity was defined as the amount of enzyme which hydrolyzed one micromol of substrate per minute.

Effects of AFLEI on fibrinogenase and collagenase activities of elastase

Elastase from *A. flavus* in 10 mM Tris-HCl buffer (pH 8.5) was incubated with AFLEI for 15 min, and with 1 mg of fibrinogen or collagen (Type I, II, III, IV) at 37°C for various time intervals. Following incubation, 0.1 m*l* of the above reaction mixture was withdrawn for an assay of clottable fibrinogen. Simultaneously, 0.1 m*l* of the reaction mixture was pipetted into a small test tube and 0.1 m*l* of buffer containing 10 M urea, 4% sodium dodecyl sulfate, 10 mM phosphate buffer (pH 7.2), and 3% β-mercaptoethanol were added. This solution was incubated at 37°C for 6 hr. An aliquot of 20 μl was then electrophoresed on a 12% SDS-polyacrylamide gel.

RESULTS

Purification of AFLEI

A sufficient number of microorganic spores were cultured in 100 m*l* of broth. Elastase inhibitory activity (100～95%) was found in 5～8 day culture supernatants. The cultures were incubated for 7 days at 37°C without shaking. Elastase inhibitory activity of the culture supernatant was measured (Fig. 1A). The culture broth was filtered, and the cell-free culture supernatant was used as the source of crude inhibitor. Solid ammonium sulfate was added (60% saturation) to 100 m*l* of the culture filtrate supernatant from *A. flavus* at room temperature. The resulting precipitate was collected by centrifugation at 3,000 r.p.m. at 10 min, and dissolved in an equal volume of the original supernatant in 10 mM Tris-HCl buffer (pH 7.5). This solution was applied on a DE52-Cellulose equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The column was eluted with equilibration buffer and 10 mM Tris-HCl buffer (pH 7.5) containing a 0.5 M NaCl gradient (Fig. 1B). Inhibitory activity (100～50%) was found in fraction numbers 170～190. The fraction numbers 170～190 were

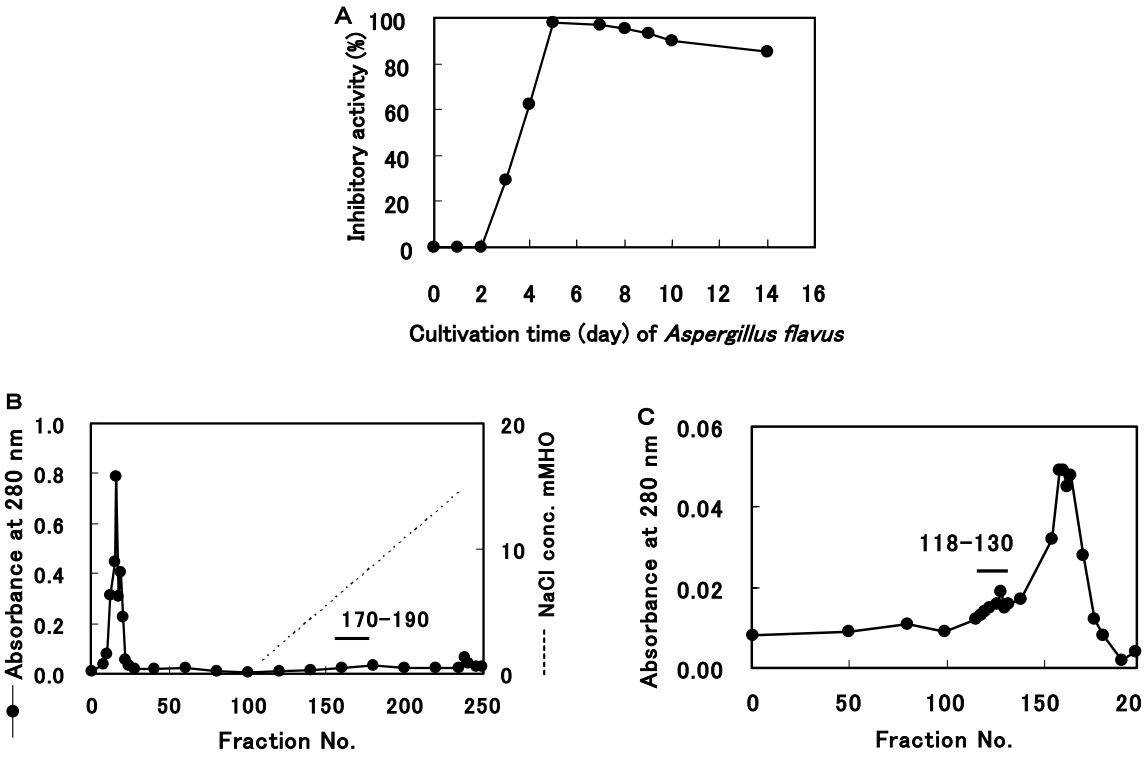

Fig. 1. Elastase inhibitory activity and column chromatography of culture filtrate from *A. flavus*.

(A) Elastase inhibitory activity of culture filtrate from *A. flavus*.
(B) DE52-Cellulose column chromatography.
(C) Sephadex G-75 column chromatography.

pooled, concentrated by lyophilization, and applied to a column of Sephadex G-75 equilibrated with 10 mM Tris-HCl buffer (pH 7.5). Inhibitory activity was found in fraction numbers 118～130 (Fig. 1C). These fraction numbers were then pooled and analyzed using polyacrylamide gel electrophoresis. AFLEI was electrophoretically homogeneous as demonstrated by polyacrylamide gel electrophoresis (pH 8.3) and isoelectric focusing/polyacrylamide gel. The yield from this purification procedure was 30 μg of AFLEI.

Properties of AFLEI

The molecular weight of the inhibitor isolated from *A. flavus* was 7,525.8 as determined by TOF-MS (Fig. 2). The pI value was found to be 7.4 by isoelectric focusing/polyacrylamide gel electrophoresis. Carbohydrate content of AFLEI was calculated to be 0% using glucose as a standard by the method of Morris[20]. The heat stability of AFLEI (0.2 μg) was investigated in 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM NaCl. The inhibitor was heated for 10 min at 37°C, 50°C, 60°C, 80°C, and 100°C, then cooled quickly to 4°C, and inhibitory activity was determined. AFLEI was stable to heat treatment (it remained 50% active at 80°C). AFLEI (0.2 μg) was incubated at 4°C for 24 hr in buffers at various pH values (100 mM acetate buffer, pH 6; 100 mM Tris-HCl buffer, pH 7, 8, 9; 100 mM sodium hydrogen carbonate buffer, pH 10) and the inhibitory activity was determined. It was stable over the pH range of 6-10.

The effect of various inhibitors on AFLEI was also determined. AFLEI was mixed with the inhibitors and incubated for 15 min at 37°C prior to the addition of STANA as substrate to determine the inhibitor effect on its inhibitory activity. AFLEI was shown to be inhibited about 60% only by α_2-macroglobulin. EDTA, benzamidine, chymostatin, TPCK and DTT did not show any inhibitory effect on the elastolytic activity of AFLEI (Table 1).

The effect of AFLEI on various elastases was determined and is shown in Table 2. Fifty microliters of AFLEI was mixed with 50 μl of the purified elastase from *A. flavus* and incubated for 15 min at 37°C. Then, 0.9 ml of 50 mM Tris-HCl buffer, pH 7.5, and 20 μl of 50 mM STANA solution were added and incubated for 60 min at 37°C. The reaction was stopped by adding 1 ml of 10% TCA. Two-tenths ml of 0.1% sodium nitrite, 0.5% ammonium sulfamate, and 0.1% N-1-naphtyletylenediamine dihydrochloride

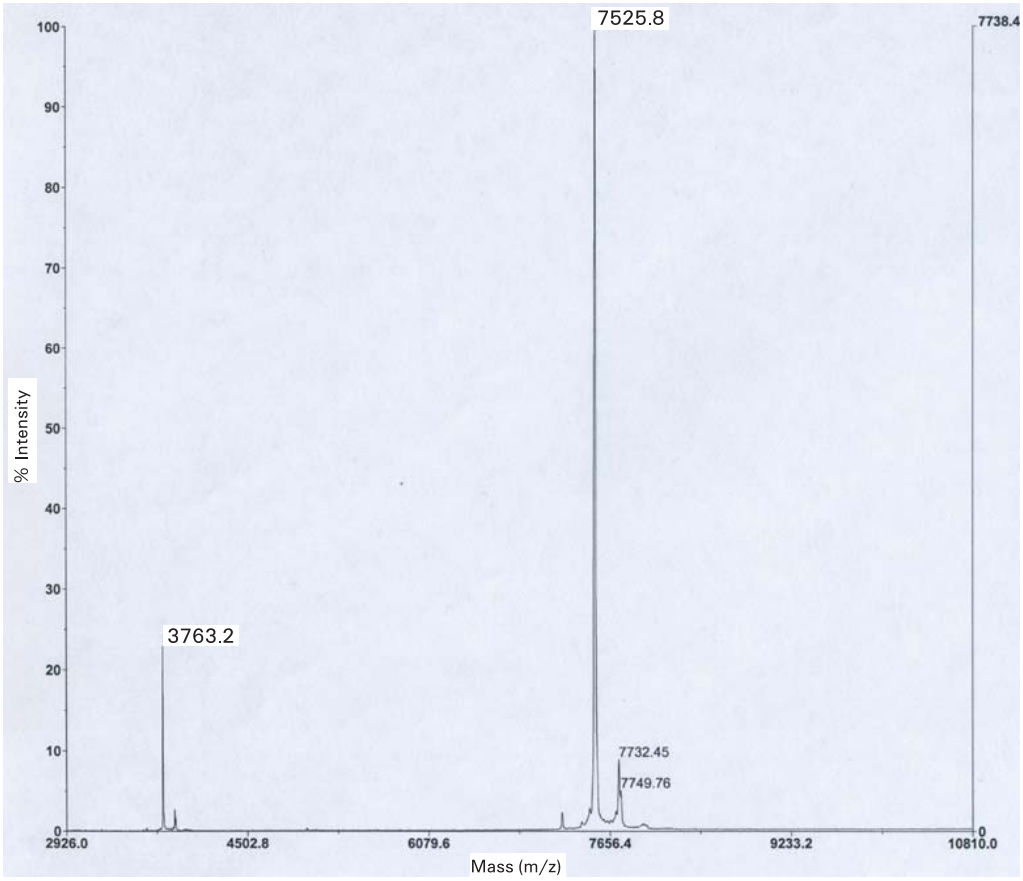

Fig. 2. Time of flight mass spectrometry of AFLEI.

Table 1. Effects of some inhibitors on AFLEI

Inhibitor	Final concentration	Residual activity (%)
None	−	100.0
α_2-macroglobulin	1 mg/m*l*	41.0
EDTA	10 mM	82.5
SBTI	5 mg/m*l*	92.6
Chymostatin	0.1 mg/m*l*	100.0
DTT	2.5 mM	100.0
Benzamidine	5 mM	99.7

EDTA: ethylenediaminetetraacetic acid-2Na
SBTI: soybean trypsin inhibitor
DTT: dithiothreitol

were then added to the solution and the color that developed was read at 550 nm. One unit is defined as the minimal inhibitory dose which did not release p-NA from STANA per minute. Elastases from *A. fumigatus*, *A. flavus* and human leukocytes were inhibited 100%, 96.1% and 72.8% by AFLEI. However, porcine pancreas elastase was not inhibited.

The effects of AFLEI on fibrinogenase and collagenase activities of elastase were measured by the method of Ouyang and Teng[21]. Both activities were completely inhibited by AFLEI.

Table 2. Inhibitory activity on some elastases and proteinases of AFLEI

Elastase and Proteinase	Inhibitory activity (%)
Purified elastase from *A. flavus*	100.0
Purified elastase from *A. fumigatus*	96.1
Purified elastase from Human leukocyte	72.8
Purified elastase from Porcine pancreas	5.5
Fibrinogenase activity of elastase from *A. flavus*	+
Collagenase activity of elastase from *A. flavus*	+
Alkaline proteinase from *P. aeruginosa*[22]	0
Ac_1-proteinase from *Agkistrodon acutus*[23]	22.3
Proteinase from *S. epidermidis*	0
Trypsin	10.2
Chymotrypsin	20.0
Thrombin	0

+) inhibition

DISCUSSION

Aspergillosis is a common mycosis in immunocompromised hosts undergoing chemotherapy. The pathogenesis of aspergillosis is not clearly understood however the literature results of this research strongly indicate that proteases are significant factors in the pathogenic process

and several of these proteases have been isolated. Frosco *et al.*[1] purified an elastase of 32,000 from *A. fumigatus* which was inhibited by EDTA. Monod *et al.*[5] purified an alkaline protease of 33,000. Rhodes *et al.*[6] purified an elastolytic protease of 23,000 from *A. flavus*. This laboratory purified an elastolytic protease from *A. fumigatus*, which had a M.W. and pI of 32,000 and 9.1, respectively[2], and proved pathogenicity of elastase. Additionally, we purified an elastolytic glycoprotein protease from *A. flavus*, which was inhibited by diisopropyl fluorophosphate. Its M.W. and pI were 40,000 and 8.6, respectively[7].

Kolattukudy *et al.*[24] purified an elastolytic serine protease of 33,000 from *A. fumigatus* and determined the primary structure by using an elastin medium. They compared the lethal activity of the elastase producing strain and non-elastase producing strain and reported that the former exhibited lethal activity. They did find that the non-elastase producing strain possessed lethal activity but at a significantly minimal level. Kothary *et al.*[10] compared the lethal activity of six strains of elastase producing and four strains of non-elastase producing *A. fumigatus* and their results were comparable to Kolattukudy *et al.*[24] Furthermore, when the dead murine pulmonary tissue was examined, it was found that elastase producing strains had more broadly invaded the tissue than non-elastase producing strains. Monod *et al.*[5] reported a similar result with an alkaline protease-deficient mutant, and Blanco *et al.*[12] presented results on the participation of elastase in pathogenicity. From these results, elastase is very important as pathogen factor.

In a previous report, screening of the elastase inhibitor from *Aspergillus* was examined[19] with the finding that *A. fumigatus* and *A. flavus* did indeed produce an elastase inhibitor. In this report and using the AFL-1 strain, both elastase activity and inhibitor activity were stronger than in the previous 13 strains used.

Development of the material which inhibits leucocyte elastase for the purpose of suppressing internal inflammation is the present focus. For example, ONO-5046[25] is used for the treatment of acute lung disturbance with a general inflammatory reaction symptom-complex. This report is the first that reveals an inhibitor capable of inhibiting leucocyte elastase.

Ogawa *et al.*[17] reported a clinical effect with a protease inhibitor. When *Aspergillus* invades the body, it must have nutrients to live. Therefore, it is thought that *Aspergillus* produces a strong elastase which breaks human tissue and supplies nutrients. Development of *Aspergillus* weakens, however, if this can be controlled and a rise in the therapeutic effect will result. The selectivity is high, molecular weight is 7,525.8, it is stable to heat and pH, and, as stated above, possesses sufficient potency to intervene in the pathogenic process of Aspergillosis.

In the future we plan to examine AFLEI in more detail by studying it *in vivo* and focusing on the possibility of using this inhibitor as another drug in the arsenal to prevent Aspergillosis.

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