Jpn. J. Med. Mycol.
Vol. 48, 13－18, 2007
ISSN 0916－4804

Original Article

Characterization and Primary Structure of Elastase Inhibitor, AFLEI from *Aspergillus flavus*

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〔Received: 22, May 2006. Accepted: 14, September 2006〕

Abstract

The amino acid sequence of elastase inhibitor, AFLEI, isolated from *Aspergillus flavus* was determined by the Edman sequencing procedure of peptides derived from digests utilizing clostripain. A molecular weight of 7,525.8 was observed by TOF-MS. AFLEI contained 68 amino acid residues and has a calculated molecular weight of 7,526.2. The search for amino acid homology with other proteins demonstrated that amino acid residues 1 to 51 of AFLEI are 100% identical to residues 20 to 70 of the hypothetical protein Afu3g14940.

The Michaelis constant (Km) for succinyl L-alanyl- L-alanyl- L-alanyl p-nitroanilide (STANA), and inhibition constant (Ki), for elastase of AFLEI, were found to be $6.7\times10^{2}\,\mu$M and $4.0\times10^{-2}\,\mu$M, respectively.

Inhibitory activity was compared with six protease inhibitors (ulinastatin, nafamostat mesilate, sivelestat sodium hydrate, gabexate mesilate, elastatinal and elafin). The other six protease inhibitors demonstrated very weak inhibitory activity by comparison with AFLEI.

Key words: *Aspergillus flavus*, elastase inhibitor, primary structure, leukocyte elastase

INTRODUCTION

Aspergillus species are the common fungi of all environments[1)]. Conidia of these fungi are airborne and found in the atmosphere throughout the world. Aspergillosis is caused by inhalation of high spore concentrations of the *Aspergillus* species and is one of the most important systemic mycoses. Invasive aspergillosis caused by pathogenic *Aspergillus* species is a serious hazard for an immunocompromised patient and the number of patients whose immune systems are compromised by either congenital or immune deficiency states has increased recently.

Elastase-producing strains promote a higher death rate in immunodeficient mice than non-elastase producing strains. Elastase-producing strains destroy lung tissue[2, 3)] and an elastase produced by *Aspergillus* is confirmed as a definitive virulence factor of Aspergillosis[4)]. Ulinastatin, which contains an enzyme inhibitor, was administered by way of intravenous drip infusion concomitantly with antifungal agents to patients of pulmonary aspergillosis[5–9)]. 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[10)]. Recently, we purified and characterized the elastase inhibitor (AFLEI) from *A. flavus*[11)]. AFLEI has a molecular weight of 7,525.8, isoelectric point of pH 7.4 and inhibits elastase elastolytic activity from *A. flavus*, *A. fumigatus* and human leukocytes. However, the elastolytic activity of porcine pancreas

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elastase, trypsin, chymotrypsin, thrombin, and Ac_1-Proteinase from snake venom was not affected. The primary structure of AFLEI was determined in order to elucidate and investigate the physiological function and biological relationship of this inhibitor. In summary, here we report the first primary structure and properties of the elastase inhibitor, AFLEI.

MATERIALS AND METHODS

Fungal strain

A. flavus (AFL-1 strain) was identified and isolated from the sputum of a patient with allergic bronchopulmonary aspergillosis. Elastase from *A. flavus* was purified by the method of Hasegawa *et al.*[12].

Purification of AFLEI

AFLEI was purified from *A. flavus* by the method of Okumura *et al.*[11]. A sufficient number of microorganic spores were cultured in 100 m*l* of synthetic medium of 0.1% yeast carbon base (Difco Lab., Detroit, MI, USA) containing 1% casamino acid (Difco Lab.) as a nitrogen source. The cultures were incubated for 7 days at 37°C without shaking. 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 rpm for 10 min, and dissolved in an equal volume of the original supernatant in 10 mM Tris-HCl buffer (pH 7.5). AFLEI was purified from this solution using DE52-cellulose (Whatman Biosystems, Ltd., England) and Sephadex G-75 (Pharmacia, Uppsala, Sweden) chromatographies.

Polyacrylamide gel electrophoresis and isoelectric focusing

Polyacrylamide gel electrophoresis (pH 8.3 and pH 4.3) was carried out according to the method of Davis[13]. Electrophoresis was carried out on polyacrylamide gel using a constant current of 3 mA at 4°C for 3 hr.

Isoelectric focusing was performed on an ampholine polyacrylamide gel. The ampholyte concentration was 4% (w/v) with a pH range of 3.5-10.5 using a constant potential of 200 V at 4°C for 4 hr.

Amino acid sequence analysis

The amino acid sequence of AFLEI was analyzed by an Applied Biosystems 491 protein sequencer. The phenylthiohydantoin (PTH) derivatives of amino acids were identified with an Applied Biosystems Model 120A PTH analyzer in accordance with the manufacturer's instructions. Native AFLEI was digested with clostripain (Roche Diagnostics K.K., Basel, Switzerland) for 24 hr at 37°C in 50 mM Tris-HCl (pH 7.2) containing 10 mM $CaCl_2$. All digests were separated by reversed-phase HPLC.

Assay for elastase inhibitory activity

Fifty microliters of AFLEI or other inhibitors (ulinastatin; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan, sivelestat sodium hydrate; Ono Pharmaceutical Co., Ltd., Osaka, Japan, gabexate mesilate; Ono Pharmaceutical Co., Ltd., nafamostat mesilate; Torii Pharmaceutical Co., Ltd., Tokyo, elafin; Peptide Institute Inc, Osaka, elastatinal; Sigma Chemical Co., Ltd., St. Louis, MO, USA) was mixed with 50 μl of the purified elastase from *A. flavus* or others (porcine pancreas; Sigma Chemical Co., Ltd and human leukocytes; Elastin Products Co., Inc., Missouri, USA). and incubated for 15 min at 37°C. Then 0.9 m*l* of 50 mM Tris-HCl buffer, pH 7.5, and 20 μl of 50 mM succinyl L-alanyl- L-alanyl- L-alanyl p-nitroanilide (STANA) (Peptide Institute Inc., Osaka) solution were added and incubated for 60 min at 37°C. The reaction was stopped by adding 1 m*l* of 10% TCA. Two-tenths m*l* of 0.1% sodium nitrite, 0.5% ammonium sulfamate, and 0.1% N-1-naphtyletylenediamine dihydrochloride were added to the solution and the color that developed was read at 550 nm.

RESULTS

Enzymatic Properties

The Michaelis constant (Km) for STANA of elastase from *A. flavus* at pH 7.5, was 6.7×10^2 μM (Fig. 1). The inhibition constant (Ki) of AFLEI for elastase was determined by

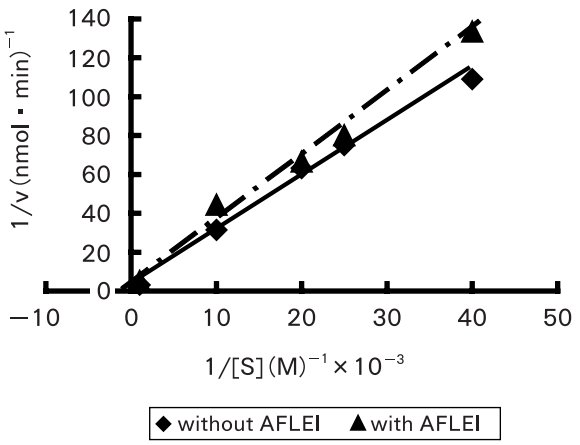

Fig. 1. Reciprocal plot of reaction velocity against concentration of STANA in the presence and absence of AFLEI.

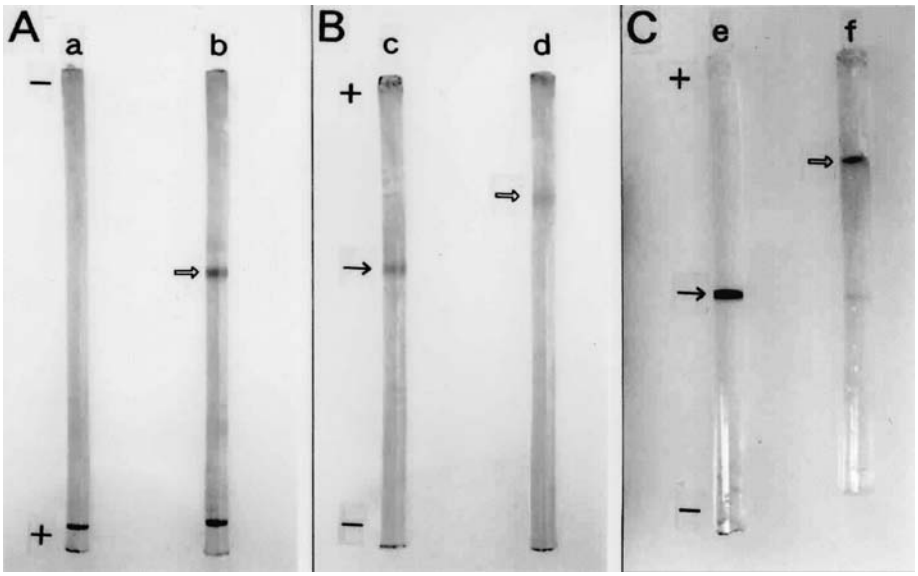

Fig. 2. Polyacrylamide gel electrophoresis (A) pH 8.3 gel electrophoresis, (B) pH 4.3 gel electrophoresis, (C) Isoelectric polyacrylamide gel electrophoresis. The black arrow is elastase from *A. flavus* (c,e), The white arrow is a mixture (elastase from *A. flavus* and AFLEI) (b,d,f). Elastase from *A. flavus* was not detected by pH 8.3 gel electrophoresis (a).

measuring the initial rate of hydrolysis of STANA at pH 7.5. The inhibition of AFLEI was non-competitive and an inhibition constant of 4.0×10^{-2} μM was obtained (Fig. 1).

An electric change of elastase

Elastase and AFLEI were mixed together and electrophoresis was carried out on polyacrylamide gel using a constant current of 3 mA at 4°C (Fig. 2). Elastase could not be detected by polyacrylamide gel electrophoresis (pH 8.3), however, the mixed sample demonstrated a single band (pH 8.3). Additionally the mixed sample migrated to the anode (pH 4.3). This suggests that AFLEI acted on elastase resulting in a change in the electricstate. This conclusion is supported by the fact that the isoelectric point became considerably lower when elastase and AFLEI were mixed and the mixed sample was tested by isoelectric point electrophoresis.

Primary structure of AFLEI

A total of 68 amino acid residues were identified (Fig. 3) and Table 1 shows the amino acid composition of AFLEI. Calculated values were derived from the result of amino acid sequence analysis. The total number of aspartic and glutamic acid residues is 9, and the combined number of lysine and arginine residues is 4. The AFLEI sequence also shows two cysteinyl residues. AFLEI was digested in clostripain and the amino acid sequence of each fragment was examined by reverse-phase high-performance

Amino acid sequence of AFLEI

D - P - A - T - C - E - K - E - A - Q -
F - V - K - Q - E - L - I - G - Q - P -
Y - T - D - A - V - A - N - A - L - Q -
S - N - P - I - R - V - L - H - P - G -
D - M - I - T - M - E - Y - I - A - S -
R - L - N - I - Q - V - N - E - N - N -
E - I - I - S - A - H - C - A

Fig. 3. Amino acid sequence of AFLEI

Table 1. Amino acid compositions of AFLEI

Amino acid	Residue
cm-Cys	2
Asp	9
Thr	3
Ser	3
Glu	11
Gly	2
Ala	8
Val	4
Met	2
Ile	7
Leu	4
Tyr	2
Phe	1
Lys	2
His	2
Arg	2
Pro	4
Trp	0
Total	68

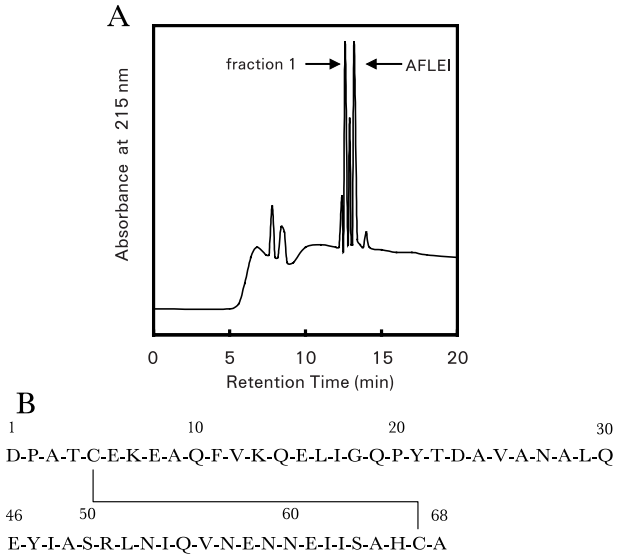

Fig. 4. (A) Fractionation by reversed-phase HPLC of the peptides obtained by clostripain cleavage of native AFLEI. Column, Develosil 300 ODS-7 column (0.46×25 cm). Solvent A, 0.1% TFA in H_2O; solvent B, 0.1% TFA in acetonitrile. Flow rate, 1.0 m*l*/min. Elution with a linear gradient from 0 to 100% in solvent B over 20 min.
(B) Amino acid sequence of fraction 1.

liquid chromatography (Fig. 4A). The sequences of fraction 1 were determined as shown in Fig. 4B. Two peptides were detected simultaneously. From these results, it was determined that one disulfide bond of AFLEI links Cys-5 to Cys-67. AFLEI is composed of 68 amino acid residues and the molecular weight of the protein portion of AFLEI was estimated to be 7,526.2. Interestingly, the search for amino acid homology with other proteins showed that amino acid residues 1 to 51 of AFLEI are 100% identical to residues 20 to 70 of the hypothetical protein Afu3g14940 (which contains 83 amino acid residues) in *A. fumigatus*[14], suggesting that although the function of Afu3g14940 protein is unknown, the protein may possess inhibitory activity for elastases. No tryptophan was found and a total of three aromatic amino acids (two tyrosines, one phenylalanine) were present. As a result, the absorption at 280 nm was minimal.

Comparison of other protease inhibitors

Inhibitory activity was compared with six other protease inhibitors (Table 2). Ulinastatin, nafamostat mesilate, sivelestat sodium hydrate, and gabexate mesilate are reagents normally used in hospitals, whereas elastatinal and elafin are laboratory reagents. AFLEI inhibited elastase from *A. flavus* and human leukocytes, but porcine pancreas elastase was not affected by AFLEI. By comparison with the other inhibitors, elastase from *A. flavus* was inhibited by elafin and human leukocyte elastase was inhibited by ulinastatin, sivelestat sodium hydrate and elafin. These results clearly demonstrate that elastase from *A. flavus* and human leukocytes were inhibited by very small amounts of AFLEI. The other six protease inhibitors demonstrated very weak inhibitory activity in comparison to AFLEI.

Table 2. Effect of various inhibitors on *A. flavus*, human leukocyte and porcine pancreas elastases

Inhibitor	Elastase		
	A. flavus	Human leukocyte	Porcine pancreas
AFLEI	0.25 μg*)	0.29 μg	>0.43 μg
Ulinastatin	>830 μg	96.34 μg	>830 μg
Nafamostat mesilate	>100 μg	100 μg	>100 μg
Sivelestat sodium hydrate	>500 μg	0.67 μg	63.13 μg
Gabexate mesilat	1707.65 μg	>2500 μg	>2500 μg
Elastatinal	>125 μg	>125 μg	7.14 μg
Elafin	44.06 μg	8.84 μg	14.45 μg

*) 50% inhibitory dose

DISCUSSION

This laboratory purified an elastolytic protease from *A. fumigatus*, which had a molecular weight of 32,000 and pI of 9.1, respectively[15], and demonstrated pathogenicity of elastase[16]. Additionally, we purified an elastolytic glycoprotein protease from *A. flavus*, which was inhibited by diisopropyl fluorophosphate. Its molecular weight and pI were 40,000 and 8.6, respectively[12]. From these results, elastases and proteases are definitely related to pathogenicity, and therefore the elastase inhibitor could be a most important treatment for aspergillosis. Most recently, we purified and characterized the elastase inhibitor (AFLEI) which had a molecular weight of 7,525.8 from *A. flavus*[11].

Ogawa *et al.*[8] 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, and if this can be controlled an elevation in the therapeutic effect will result. Elastase from *A. flavus* was inhibited by a very small quantity of AFLEI in comparison with conventional protease inhibitors. Umezawa *et al.*[17] obtained elastatinal from *Actinomycetes*, Ohno *et al.*[18] obtained elasnin from *Streptomyces noborioensis* KM-2753, Sato and Murao[19] obtained S-SI from *Streptomyces albogriseolus* S-3253, Dubin *et al.*[20] obtained inhibitor leucocyte cells and Wiedow *et al.*[21] derived elafin from human skin. In this

way various elastase inhibitors have been derived from various places and the purpose of these elastase inhibitors has been examined. Most of these inhibitors are presently in use in clinics or are being marketed as inhibitor reagents. AFLEI showed the strongest inhibition compared with the six protease inhibitors used in this experiment. Since AFLEI strongly inhibited human leucocyte elastase, it can be expected to have significant effects in the prevention of tissue disorder.

When *Aspergillus* invades the body, elastase is produced by the leucocytes, which continues to damage tissue and to supply nutrients to support *Aspergillus*. If AFLEI can inhibit these elastases, development of *Aspergillus* and histionic breakdown are prevented. Furthermore, it is thought that AFLEI can suppress the onset of *Aspergillus* for a long period when it is used in combination with an immunosuppressive agent.

ACKNOWLEDGEMENTS

This research was financially supported by a Grant-in-Aid for a High-Tech Research Center Project from the Ministry of Education, Culture, Sports, Science and Technology of Japan, which is gratefully acknowledged. We are thankful to Ayumi Kondou, Masayo Iguchi, Natsu Sato and Chisato Sugimura for technical support.

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