Biochemical properties and primary structure of elastase inhibitor AFUEI from *Aspergillus fumigatus*

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An elastase inhibitor from *Aspergillus fumigatus* (AFUEI) was isolated, and its biochemical properties and primary structure examined. The inhibitor was purified by column chromatography using DE52 cellulose and Sephadex G-75, and was found to be homogeneous as indicated by a single band following discontinuous PAGE and SDS-PAGE. A molecular mass of 7525.1 Da was observed by matrix-assisted desorption/ionization time-of-flight mass spectroscopy. The elastolytic activity of elastases from *A. fumigatus*, *Aspergillus flavus* and human leukocytes was inhibited by AFUEI. However, the elastolytic activity of porcine pancreas elastase, *Pseudomonas aeruginosa* elastase and elastase from snake venom was not affected by AFUEI. No inhibitory effect of DTT or 2-mercaptoethanol on the elastase inhibitory activity of AFUEI was observed. The amino acid sequence of AFUEI peptides derived from digests utilizing clostripain was determined by Edman sequencing. AFUEI was composed of 68 aa and had a calculated molecular mass of 7526.2 Da. The search for amino acid homology with other proteins demonstrated that aa 1–68 of AFUEI are 100% identical to aa 20–87 of the hypothetical protein AFUA 3G14940 of *A. fumigatus*.

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INTRODUCTION

Currently, the genus *Aspergillus* has been shown to comprise more than 150 species. Approximately ten species have been isolated from clinical material, in particular *Aspergillus fumigatus*. Aspergillosis is a common mycosis occurring in immunocompromised hosts undergoing chemotherapy. It is caused by inhalation of high concentrations of spores of the species *Aspergillus*. It has been reported that various proteases are produced by *A. fumigatus* (Frosco *et al.*, 1992b; Hasegawa *et al.*, 1995; Larcher *et al.*, 1992; Monod *et al.*, 1991, 1993), *Aspergillus flavus* (Hasegawa *et al.*, 1997; Rhodes *et al.*, 1990) and *Aspergillus niger* (Inoue *et al.*, 1991; Takahashi *et al.*, 1991). As 28% of lung tissue is composed of elastin, a strong correlation between elastase and the pathogenesis of aspergillosis has been suggested.

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 (Kothary *et al.*, 1984; Reichard *et al.*, 1990), and an elastase produced by *Aspergillus* has been confirmed as a virulence factor of aspergillosis (Blanco *et al.*, 2002). We have previously studied and reported the pathogenicity of elastase produced by *A. flavus* in mice immunocompromised with cyclophosphamide, cyclosporine, prednisolone and carrageenan. These studies suggested that, when infected with *A. flavus*, a severe lesion in bronchioli or alveoli is induced by elastase-producing strains (Okumura *et al.*, 2007a).

Ulinastatin, an elastase inhibitor, has been administered by intravenous drip infusion concomitantly with antifungal agents to patients with pulmonary aspergillosis (Hasegawa *et al.*, 1994; Maesaki *et al.*, 1993; Ogawa, 1997; Ogawa *et al.*, 1992; Sugimura *et al.*, 1994) and it has been suggested that this elastase inhibitor could be an effective therapeutic agent for aspergillosis. It has been shown that *A. fumigatus* and *A. flavus* produce elastase inhibitors (Okumura *et al.*, 2004). We previously reported the isolation and the primary structure of an elastase inhibitor from *A. flavus* (Okumura *et al.*, 2007b). In this paper, we report the isolation, biochemical properties and primary structure of

Abbreviations: GAAPLNA, glutaryl-L-alanyl-L-alanyl-L-prolyl-L-leucine *p*-nitroanilide; MALDI/TOF-MS, matrix-assisted desorption/ionization time-of-flight mass spectroscopy; Pe-AFUEI, S-pyridylethylated AFUEI; pNA, *p*-nitroanilide.

an elastase inhibitor from *A. fumigatus* (strain AFU-12) from a patient.

METHODS

Strain. *A. fumigatus* strain AFU-12 was identified and isolated from the sputum of a patient with allergic bronchopulmonary aspergillosis. Elastases from *A. fumigatus* and *A. flavus* were purified using the method of Hasegawa *et al.* (1995, 1997).

Production of elastase inhibitor. Conidia (5×10^6) were cultured in 100 ml synthetic medium comprising 0.1% yeast carbon base (Difco) with 1% casamino acid (Difco) as a nitrogen source. The cultures were incubated for 7 days at 37 °C. The elastase inhibitory activity of the culture supernatant was measured (Fig. 1) and determined to be approximately 65–90% in culture supernatants of 4–7 days. The 7 day culture broth was filtered aseptically through a 0.22 µm membrane filter and the cell-free culture supernatant was used as the crude source of inhibitor.

Discontinuous PAGE. Discontinuous PAGE was carried out on 8.5% polyacrylamide gels (0.5 × 12.5 cm, 3 mA, 4 °C), using 40 mM Tris/glycine (pH 8.3) as the running buffer. Gels were stained with 0.05% Coomassie brilliant blue R-250 in 1% acetic acid containing 5% methanol for 1 h at 48 °C, followed by diffusion destaining.

Purification of AFUEI. The 7 day culture broth was applied to a column of diethylaminoethyl (DE52) cellulose (column size 2 × 45 cm; Whatman) equilibrated with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. The column was eluted with 10 mM Tris/HCl (pH 7.2) containing a 0–0.5 M NaCl gradient (Fig. 2a). Inhibitory activity (100–50%) was found in fractions 232–300, which were pooled, concentrated by lyophilization and applied to a Sephadex G-75 column (2.5 × 100 cm; Pharmacia) equilibrated with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. Inhibitory activity of 100% was found in fractions 115–130 (Fig. 2b). These fractions were analysed for purity by PAGE.

Assay for elastolytic inhibitory activity. The elastolytic inhibitory activity of AFUEI was assayed by the method of diazo coupling. Using 50 mM glutaryl-L-alanyl-L-alanyl-L-prolyl-L-leucine *p*-nitroanilide (GAAPLNA; Peptide Institute) in DMSO as the substrate, the amount of *p*-nitroanilide (pNA) released was measured. Fifty microlitres of AFUEI (3 ng) was mixed with 50 µl elastase (30 ng) and the mixture was incubated for 15 min at 37 °C. Next, 900 µl

Fig. 1. Elastase inhibitory activity of *A. fumigatus* culture filtrate.

Fig. 2. Column chromatography of *A. fumigatus* culture filtrate. (a) DE52 cellulose column chromatography. *A. fumigatus* culture filtrate (100 ml) was applied to a DE52 cellulose column (2 × 45 cm) equilibrated with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. The column was eluted with 10 mM Tris/HCl (pH 7.2) containing a 0–0.5 M NaCl gradient. Fractions (6 ml) were collected at a flow rate of 18 ml h⁻¹. (b) Sephadex G-75 column chromatography. DE52 cellulose column fractions 232–300 were pooled, concentrated by lyophilization and applied to a Sephadex G-75 column (2.5 × 100 cm) equilibrated with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. The column was eluted with 10 mM Tris/HCl (pH 7.2) containing 10 mM NaCl. Fractions (3 ml) were collected at a flow rate of 9 ml h⁻¹. ●, A₂₈₀; ○, inhibitory activity; dashed line, NaCl concentration.

50 mM Tris/HCl (pH 7.5) and 20 µl 50 mM GAAPLNA solution were added and incubated for 60 min at 37 °C. The reaction was stopped by adding 1 ml 10% (w/v) trichloroacetic acid. Sodium nitrite (0.2 ml, 0.1%), 0.2 ml 0.5% ammonium sulfamate and 0.2 ml 0.1% N-1-naphthylethylenediamine dihydrochloride were then added to the solution and the absorbance was read at 550 nm. One unit of elastolytic inhibitory activity was defined as the amount of AFUEI (mg) that did not release pNA from GAAPLNA in 1 min.

Effects of AFUEI on fibrinogenase and collagenase activities of elastase. Elastase from *A. fumigatus* in 10 mM Tris/HCl (pH 8.5) was incubated with the AFUEI supernatant for 15 min and then with 1 mg fibrinogen (Katayama Chemical) or collagen (types I, II, IV and V; Wako Pure Chemical) at 37 °C for various time intervals. Simultaneously, 100 µl of the reaction mixture was pipetted into a small test tube and 100 µl buffer containing 10 M urea, 4% SDS, 10 mM phosphate buffer (pH 7.2) and 3% 2-mercaptoethanol were added. This solution was incubated at 37 °C for 6 h. An aliquot of 20 µl was analysed by 12% SDS-PAGE.

Enzymic cleavage. Native AFUEI (40 µg) was digested with 5 µg clostripain (Roche) for 24 h at 37 °C in 50 mM Tris/HCl (pH 7.2) containing 1 % (v/v) 2-mercaptoethanol and 10 mM CaCl₂. Native AFUEI was dissolved in 1.6 ml 5 mM Tris/HCl (pH 7.5) containing 8 M urea, and 200 µl 2-mercaptoethanol was added to this solution. The reaction mixture was bubbled through with nitrogen for 10 min and incubated at 37 °C for 1 h. 4-Vinylpyridine (200 µl) was added and the reaction mixture was stirred at room temperature for another 2 h. The excess reagents were removed by dialysis against distilled water. S-Pyridylethylated AFUEI (Pe-AFUEI) was also digested with clostripain as above. All digests were then separated by reversed-phase HPLC using a Gilson model 302 chromatograph with a Develosil 300 ODS-HG5 column (0.46 × 25 cm).

Sequence analysis. The amino acid sequence of AFUEI was analysed using an Applied Biosystems 491 protein sequencer. The phenylthiohydantoin derivatives of amino acids were identified using an Applied Biosystems model 120A PTH analyser in accordance with the manufacturer's instructions.

Determination of molecular mass. AFUEI was desalted using a ZipTip column (Millipore) and analysed by matrix-assisted desorption/ionization time-of-flight mass spectroscopy (MALDI/TOF-MS) using a Voyager PK2 (Applied Biosystems).

RESULTS AND DISCUSSION

AFUEI isolation

AFUEI was isolated from *A. fumigatus* by DE52 cellulose and Sephadex G-75 column chromatography as described in Methods. Fractions 115–130 (Fig. 2b) were analysed by discontinuous PAGE (pH 8.3) and SDS-PAGE (Fig. 3), and were found to be electrophoretically homogeneous. These fractions were pooled, and this preparation was designated the purified AFUEI (elastase inhibitor from *A. fumigatus*), which was used for the biochemical characterization. The total yield of purified AFUEI from 100 ml culture supernatant was 97 µg.

Properties of AFUEI

The molecular mass of the inhibitor isolated from *A. fumigatus* was 7525.1 Da as determined by MALDI/TOF-MS (Fig. 4). The carbohydrate content of AFUEI was calculated to be 0 % with glucose as a standard using the method of Morris (1948). The heat stability of AFUEI (0.1 µg) in 10 mM Tris/HCl (pH 7.5) containing 10 mM NaCl was investigated. The inhibitor was heated for 10 min at 37, 50, 60, 80 and 100 °C, cooled quickly to 4 °C and the inhibitory activity was determined. AFUEI was stable to heat treatment, retaining 33.3 % activity after treatment at 100 °C. The effect of reducing agents on AFUEI was also determined. AFUEI was mixed with the reducing agent and incubated for 15 min at 37 °C prior to the addition of GAAPLNA as substrate to determine whether reduction had an effect on its inhibitory activity. AFUEI activity was not inhibited by 2.5 mM DTT or 1.9 mM 2-mercaptoethanol.

The effect of AFUEI on various elastases was determined and is shown in Table 1. Elastases from *A. fumigatus*,

Fig. 3. SDS-PAGE of purified AFUEI. SDS-PAGE was performed using the method of Weber & Osborn (1969). Protein standards used were insulin B chain (3.5 kDa), aprotinin (6 kDa), lysozyme (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), BSA (66 kDa), phosphorylase b (97.4 kDa) and myosin (200 kDa). AFUEI was treated with 4.3 % SDS, reduced with 10 % 2-mercaptoethanol for 3 min at 100 °C and then run on a polyacrylamide gel (MULTIGEL II Mini 15/25; Daiichi Pure Chemicals).

A. flavus, human leukocytes and porcine pancreas were inhibited 100, 98.9, 83.8 and 11.7 % by AFUEI, respectively. However, *Pseudomonas aeruginosa* (Komori *et al.*, 2001) and snake venom elastases were not inhibited. The effects of AFUEI on the fibrinogenase and collagenase activities of elastase from *A. fumigatus* were measured. Both activities of the elastase were completely inhibited by AFUEI. The Michaelis constant (K_m) for GAAPLNA of elastase from *A. fumigatus* at pH 7.5 was 3.8×10^2 µM. The inhibition constant (K_i) of AFUEI for elastase was determined by

Fig. 4. MALDI-TOF MS results for AFUEI.

Table 1. Inhibitory activity of AFUEI on elastases

Elastase/proteinase	Substrate	Inhibitory activity (%)
Purified elastase from <i>A. fumigatus</i>	GAAPLNA	100.0
Purified elastase from <i>A. flavus</i>	GAAPLNA	98.9
Purified elastase from human leukocytes	SAPANA	83.8
Purified elastase from porcine pancreas	STANA	11.7
Elastase from <i>P. aeruginosa</i>	GAAPLNA	0
Elastase from <i>Crotalus atrox</i> venom	STANA	1.7
Fibrinogenase activity of elastase from <i>A. fumigatus</i>	Fibrinogen	IN
Collagenase activity of elastase from <i>A. fumigatus</i>	Collagen (types I, II, IV and V)	IN

IN, Complete inhibition; SAPANA, succinyl-Ala-Pro-Ala-pNA; STANA, succinyl-Ala-Ala-Ala-pNA.

measuring the initial rate of hydrolysis of GAAPLNA at pH 7.5. The inhibition of AFUEI was non-competitive and an inhibition constant of 1.6×10^{-3} μ M was obtained.

Primary structure of AFUEI

The first 44 residues of AFUEI were determined by N-terminal sequence analysis (Fig. 5). AFUEI was incubated with clostripain. The digested fragments were purified by reversed-phase HPLC (Fig. 6a) and their sequences analysed (Table 2). Pe-AFUEI was also incubated with clostripain and analysed by reversed-phase HPLC (Fig. 6b), and the amino acid sequence of each fragment (Pe-R-1 and Pe-R-2) was examined (Fig. 5). The AFUEI sequence showed two cysteinyl residues. From these results, a total of 68 aa residues of AFUEI were identified (Fig. 5). Thus, AFUEI is composed of 68 aa and the molecular mass of the protein portion of AFUEI was estimated to be 7526.2 Da. The molecular mass of AFUEI based on the amino acid sequence was identical to the molecular mass obtained by MALDI/TOF-MS. The hypothetical protein AFUA 3G14940 of *A. fumigatus* has been reported (Nierman *et al.*, 2005) and its amino acid sequence from aa 20 to 87

was found to be identical to aa 1–68 of AFUEI. It is likely that this hypothetical protein also has an inhibitory activity. No tryptophan residue was found and a total of three aromatic amino acids (two tyrosines and one phenylalanine) were present. As a result, the absorption at 280 nm was minimal (Fig. 2b).

Comparison with other protease inhibitors

Comparative studies of the inhibitory activity of AFUEI with four other protease inhibitors were performed (Table 2). Ulinastatin, nafamostat mesilate, sivelestat sodium hydrate and gabexate mesilate are proteinase inhibitors normally used for the treatment of acute pancreatitis, acute lung disturbance and intravascular coagulation disease. Our results showed that AFUEI inhibited elastase from *A. fumigatus*. Inhibition by nafamostat mesilate was also observed; however, all four protease inhibitors demonstrated very weak inhibitory activity in comparison with AFUEI. These results clearly demonstrated that AFUEI has a strong inhibitory effect on elastase from *A. fumigatus* and that only low concentrations of AFUEI are needed for inhibition.

Aspergillosis is a common mycosis occurring in immunocompromised hosts undergoing chemotherapy. The pathogenesis of aspergillosis is not clearly understood. However, the published results on pathogenesis strongly indicate that proteases are significant factors in the pathogenic process. Several of these proteases have been isolated: Frosco *et al.* (1992a) purified an elastase of 32 000 Da from *A. fumigatus* that was inhibited by EDTA, Monod *et al.* (1991) purified an alkaline protease of 33 000 Da, Rhodes *et al.* (1990) purified an elastolytic protease of 23 000 Da from *A. flavus*, and Kolattukudy *et al.* (1993) purified an elastolytic serine protease of 33 000 Da from *A. fumigatus* and determined the primary structure by using an elastin medium. The latter study compared the lethal activity of the elastase-producing and non-elastase producing strains and reported that the former exhibited lethal activity. They also found that the non-elastase-producing strain possessed lethal activity, but this was at a negligible minimum level. Kothary *et al.* (1984) compared the lethal activity of six elastase-producing strains and four non-elastase-producing

Fig. 5. Amino acid sequence of AFUEI. Arrows indicate residues determined by sequence analysis.

Fig. 6. Reversed-phase HPLC fractionation of the peptides obtained by clostripain cleavage of AFUEI. Native AFUEI (a) or Pe-AFUEI (b) was incubated with clostripain and aliquots were taken for analysis by reversed-phase HPLC using a Develosil 300 ODS-HG-5 column (0.46×25 cm). Solvent A was 0.1 % trifluoroacetic acid (TFA) in H₂O, solvent B was 0.1 % TFA in acetonitrile and the flow rate was 1.0 ml min⁻¹. Elution was achieved over 60 min with a linear gradient from 0 to 100 % using solvent B.

strains of *A. fumigatus*. Their results were comparable to those of Kolattukudy *et al.* (1993). Furthermore, when the dead murine pulmonary tissue was examined, it was found that elastase-producing strains had broadly invaded the tissue more than non-elastase-producing strains. Monod *et al.* (1991) reported a similar result with an alkaline protease-deficient mutant, and Blanco *et al.* (2002) presented results on the participation of elastase in pathogenicity. As lung tissue contains large amounts of elastin, we focused on elastase as a factor of infection. Our laboratory purified an elastolytic protease from *A.*

fumigatus with a molecular mass of 32 000 Da and pI of 9.1 (Hasegawa *et al.*, 1995) that has been shown to cause pathogenicity. Additionally, we purified an elastolytic glycoprotein protease from *A. flavus* that was inhibited by diisopropyl fluorophosphate. This protease had a molecular mass of 40 000 Da and pI of 8.6 (Hasegawa *et al.*, 1997) and has also been shown to cause pathogenicity (Okumura *et al.*, 2007a). It is clear from these results that elastases are related to pathogenicity and therefore that an elastase inhibitor could be an important treatment for aspergillosis.

In a previous report, screening of the elastase inhibitor from *Aspergillus* was conducted and it was found that both *A. fumigatus* and *A. flavus* produced an elastase inhibitor (Okumura *et al.*, 2004). In addition, both the elastase activity and inhibitory activity of the AFU-12 strain were found to be stronger than those in the previous 13 strains used. In this report, the elastase inhibitor from *A. fumigatus* (AFUEI) was purified, and its biochemical properties and primary structure examined.

Development of a substance that inhibits leukocyte elastase for the purpose of suppressing internal inflammation is a current focus. For example, ONO-5046 (Kawabata *et al.*, 1991) is used for the treatment of acute lung disturbance with a general inflammatory reaction symptom complex. The results of our studies reveal an inhibitor capable of inhibiting leukocyte elastase. AFUEI is therefore a promising agent for suppressing inflammatory diseases effectively, as it is a potent inhibitor of human leukocyte elastase.

Elastase from *A. fumigatus* displays strong degrading effects on fibrinogen and collagen (types I, II, IV and V). AFUEI strongly inhibited the activity of the elastase and therefore could prevent the degrading effect of *A. fumigatus* on fibrinogen and collagen. Type IV collagen is the main component of the basal membrane of blood vessels and alveoli, and the bleeding seen on histopathological examination is thought to be caused by injury to the basal membrane of these structures. Moreover, elastase has an injurious effect locally at the infection site, and is thought to be related in some way to the pathogenicity manifested by the tissue infiltration and inflammation exhibited by *A. fumigatus*. Taken together, these findings suggest that AFUEI inhibits tissue infiltration and inflammation seen with aspergillosis and thereby weakens pathogenicity. Ogawa *et al.* (1992) reported a clinical effect with the protease inhibitor ulinastatin. When ulinastatin was administered to a patient with lung aspergillosis who had recurrent haemoptysis and haemospitum, the symptoms disappeared within a short period. Maesaki *et al.* (1993) and Sugimura *et al.* (1994) reported that cases of pulmonary aspergilloma and invasive pulmonary aspergillosis were successfully treated with the combined use of ulinastatin and amphotericin B.

The elastase inhibitor from *A. flavus* (AFLEI) has been purified and its biochemical properties and primary structure examined previously (Okumura *et al.*, 2006,

Table 2. Effect of various inhibitors on elastase from *A. fumigatus*

Inhibitor	IC ₅₀ (μg)
AFUEI	0.0026
Ulinastatin	>830
Nafamostat mesilate	72
Sivelestat sodium hydrate	>500
Gabexate mesilate	2000

2007b). The biochemical properties and primary structure of AFUEI were identical to AFLEI. It is interesting that *A. fumigatus* and *A. flavus* produce elastase inhibitors with the same primary structure. Most cases of aspergillosis are caused by *A. fumigatus* and *A. flavus*. If AFUEI inhibits elastases from *A. fumigatus* and *A. flavus in vivo*, it is predicted that AFUEI will be an effective agent against most cases of aspergillosis.

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