Original Research Article

Characterization and Cytotoxicity of Elastolytic Protease, Asnilase from *Aspergillus nidulans*

ABSTRACT

Aims: To elucidate the pathogenicity of *Aspergillus nidulans*, elastolytic protease was isolated from the culture supernatant of the isolate, and its biological activity and cytotoxicity were examined.
Methodology: *A. nidulans* (NBRC 4340) spores were cultured on YCB-elastin medium for three days and elastolytic protease was isolated from the culture supernatant by DEAE-Cellulose anion exchange chromatography. Elastolytic activity was measured by using GAAPLNA (Glutaryl-L-alanyl-L-alanyl-L-prolyl-L-leucine *p*-nitroanilide) as the substrate. Molecular mass and isoelectric point were determined by polyacrylamide gel electrophoresis. Substrate specificities were measured by using fibrinogen, collagen, oxidized insulin B chain. Cytotoxicity of Asnilase on human pulmonary artery endothelial cells (HPAEC), human small airway epithelial cells (HSAEC), human bronchial/tracheal epithelial cells (HBTEC) and human pulmonary alveolar epithelial cells (HPAEpiC) was determined with colorimetric method.
Results: An elastolytic protease isolated from the culture supernatant of *A. nidulans* was found to be homogeneous as indicated by a single band after polyacrylamide gel electrophoresis (PAGE), and the final preparation named as Asnilase. Molecular mass of this enzyme was determined to be 33,800 Da and the isoelectric point was 4.2. Asnilase cleaved the Aα and Bβ chains of fibrinogen, collagen and hydrolyzed His(5)-Leu(6) and Glu(13)-Ala(14) bonds of oxidized insulin B chain. Cytotoxic effects for various human pulmonary cells were observed when Asnilase was added at concentration of 2.0-8.0 μg/mL.
Conclusion: It is shown in our current investigation that Asnilase is a newly isolated elastolytic protease from *A. nidulans*. It is responsible for pulmonary aspergillus infection.

Keywords: Aspergillus nidulans, *elastolytic protease*, *proteolytic specificity*, *cytotoxicity.*

1. INTRODUCTION

Aspergillosis is one of the opportunistic infectious diseases caused by fungus, and patients with aspergillosis increase with the development of the medical technology. Invasive pulmonary aspergillosis, a common mycosis in immunocompromised hosts undergoing chemotherapy, is caused by inhalation of high concentrations of spores from *Aspergillus* species, and becomes a significant public health problem. Several species of *Aspergillus* are responsible for the diseases, aspergillosis. They are *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus* [1, 2]. *A. nidulans* is a typical soil fungus with a world-wide distribution and is also a causative agent of aspergillosis in humans and animals [1].

Correlation of elastolytic protease produced by *Aspergillus* species with ability to cause pulmonary invasive aspergillosis has been discussed [3-6]. Elastin fibres are found in vertebrate tissues and essential to maintain shape and mechanical rigidity. Since human lung is elastin-rich tissue [7], the destruction of the elastin is thought to be involved in pathogenicity. Various extracellular serine proteases and elastolytic proteases were purified from *A. fumigatus* that was most common species isolated from invasive aspergillosis, and their properties have been reported [8-12]. Although the presence of protease in different strains of *A. flavus*, *A. terreus*, *A. parasiticus* and *A. nidulans* were also reported, however, very few studies were made whether the enzyme was related to pathogenesis [13-18]. Therefore, the purified elastolytic enzyme was injected into lung tissues directly to see any pathogenic effect of these enzymes [19, 20]. It was found from the present study that the enzyme induced the inflammatory cells to accumulate in the lung and hemorrhagic pneumonia was produced. In this paper, we report biochemical properties of novel elastolytic protease from *A. nidulans* which was named as Asnilase, and investigations of its injury effects on cultural cells.

2. MATERIAL AND METHODS

2.1 Materials

A. nidulans NBRC 4340 was obtained from National Institute of Technology and Evaluation Biological Resource Center. Yeast carbon base (YCB) was purchased from Difco Lab. and DEAE-Cellulose was from Whatman Inc. A standard kit for the molecular weight determination was purchased from Sigma-Aldrich Co. LLC., pI marker was from SERVA Electrophoresis GmbH, and Bio-lyte (pH 3.5-10) was obtained from Bio-Rad Laboratories. Inc. Human fibrinogen was supplied by Sigma-Aldrich Co., Ltd. Collagen types I, II, III, and IV were obtained from Wako Pure Chemical Industries, Ltd. Glt-Ala-Ala-Pro-Leu-*p*NA (GAAPLNA), succinyl L-alanyl-L-alanyl-L-alanyl *p*-nitroanilide (STANA), and succinyl L-alanyl-prolyl-L-alanyl *p*-nitroanilide (SAPANA) were the products from Peptide Institute Inc. Elastin, azocasein, azoalbumin, azocollagen, hide powder azure, Suc(OMe)-Ala-Ala-Pro-Val-pNA and oxidized insulin B chain were purchased from Sigma-Aldrich Co. LLC. Cryo-preserved human pulmonary artery endothelial cells (HPAEC), human small airway epithelial cells (HSAEC), human bronchial/tracheal epithelial cells (HBTEC), their respective cell culture medium (HuMedia EG-2, BronchiaLife™ SAE Comp Kit and BronchiaLife™ B/T Comp Kit) and other cell culture supplements and reagents were the products of Kurabo (Osaka, Japan). Cryo-preserved human pulmonary alveolar epithelial cells (HPAEpiC) and its respective culture medium were purchased from ScienCell Research Lab. Cell Counting Kit-8 was obtained from Dojindo Lab. (Kumamoto, Japan) and CellTracker™ Green fluorescent probe was from Lonza Japan. Other chemicals used were of analytical grade and purchased from commercial sources.

2.2 Production of elastolytic protease

A sufficient number of *A. nidulans* spores were cultured in 100 ml YCB-elastin medium (a synthetic medium of 1% yeast carbon base containing 1 % elastin) as a nitrogen source. The cultures were incubated for 3 days at 37℃ with shaking. The cult ure broth was filtered through a 0.22 µm pore size membrane filter, and the cell-free culture supernatant was used as the source of crude enzyme.

2.3 Isolation and characterization of elastolytic proteinase

One hundred milliliters of the culture filtrate from *A. nidulans* was applied on a DEAE-Cellulose equilibrated with 10 mM Tris-HCl buffer (pH 7.2) containing 10 mM NaCl. The column was eluted with equilibration buffer and 10 mM Tris-HCl buffer (pH 7.2) containing 0.5 M NaCl.
The molecular mass of the enzyme was determined by SDS-PAGE [21], and the isoelectric point (pI) was estimated by isoelectric focusing/polyacrylamide gel electrophoresis using bio-lyte concentration of 40 % (w/v) with a pH range of 3.5-10. Protein concentration was measured by the method of Bradford [22] using bovine serum albumin as the standard.

2.4 Assay for elastolytic activity

Elastolytic activity of the enzyme was assayed by the diazo coupling method that measuring *p*-nitroanilide (*p*-NA) released from the synthetic substrate [23]. Briefly, 0.1 ml of the enzyme solution, 20 µl of 50 mM GAAPLNA solution dissolved in DMSO and 0.9 ml 50 mM Tris-HCl buffer (pH 7.5) were incubated for 60 minutes at 37℃. The reaction was stopped by adding 1 ml of 10 % trichloroacetic acid. Then 0.2 ml of 0.1 % sodium nitrate, 0.2 ml of 0.5 % ammonium sulfamate, and 0.2 ml of 0.1 % *N*-1-naphthylethylenediamine dihydrochloride were added to the reaction mixture, and the amount of *p*-nitroaniline released was quantified by measuring the absorbance at 550 nm. One unit of GAAPLNA hydrolase activity was defined as the amount of enzyme that hydrolyzed 1 µmol of substrate per minute.
The elastolytic activity was assayed at 19, 37, 45, 50 and 60℃, and optimum temperature of Asnilase was determined. The optimum pH was measured with 50 mM acetate buffer (pH 6.0), 50 mM Tris-HCl buffer (pH 7.5 and 8.0). The heat stability and pH stability of Asnilase was investigated as follows: The enzyme was incubated at 37, 50, 60, 70, and 80℃ fo r 10 minutes in 10 mM Tris-HCl buffer (pH 7.5) or incubated at 4℃ for 24 hours in buffers at vari ous pH values –100 mM acetate buffer (pH 5.0, 6.0), 100 mM Tris-HCl buffer (pH 7.0, 8.0, 9.0) and 100 mM sodium hydrogen carbonate buffer (pH 10.0, 12.0) – and then, the residual elastolytic activity was measured.

The effects of inhibitors on elastolytic activity were determined after the incubation of enzyme with inhibitor at 37℃ for 10 minutes.

2.5 Other enzyme activities

Fibrinogenase or collagenase activities of the enzyme were measured by monitoring the time course degradation of these proteins on SDS polyacrylamide gel. Protease activity was assayed using keratin, hide powder azure, azocasein and azoalbumin as a substrate [24-26]. Oxidized insulin B chain was used for determination of enzyme specificity. Briefly, the hydrolyzed peptides were fractionated by a reversed-phase HPLC column (Develosil 300 ODS-7), and the fragments were identified using Applied Biosystems 491 protein sequencer and 120A PTH analyzer.

2.6 Cytotoxicity assay

Cultured human cells were trypsinized, re-suspended in the medium and seeded into 96-multiwell plates at a density of 1.5×10^4 cells/well. Purified enzyme was diluted in sterilized saline at an appropriate concentration and then added to the cells and incubated at 37℃ for 24 hours. The viable cell numbers were determined by the colorimetry by means of a cell counting kit based on the tetrazolium salt/formazan system [27, 28]. For fluorescence microscopy, cell staining was performed with 0.5 mM of 5-chloromethyl-fluorescein diacetate, CellTracker™ Green CMFDA in serum-free medium according to the instruction of supplier. Fluorescence microscope system, BZ-X700 (Keyence, Osaka, Japan) was used for the observation.

3. RESULTS AND DISCUSSION

3.1 Isolation and characterization of elastolytic protease

The adsorbed fraction of culture filtrate which was eluted from DEAE-Cellulose column with 0.5M NaCl contained elastolytic protease, and the enzyme activity was found in the fractions from 210 to 240 (Figure 1). The fraction with elastolytic activity was homogeneous with various tests such as polyacrylamide gel electrophoresis, isoelectric focusing and SDS-PAGE. This protein is named as "Asnilase" (elastase from *A. nidulans*). Approximately 1.7 mg of Asnilase was obtained from 100 mL of the culture filtrate,

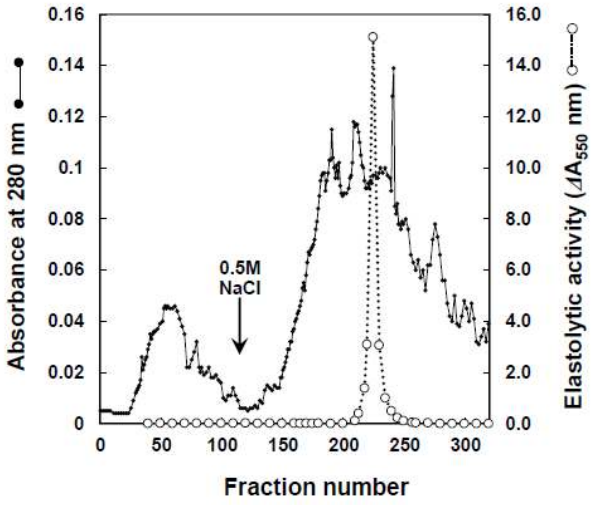

Fig. 1. DEAE-Cellulose column chromatography of culture filtrate from *A. nidulans*

One hundred milliliters of the culture filtrate was applied on a DEAE-Cellulose column (2.2×45 cm) equilibrated with 10 mM Tris-HCl buffer (pH7.2) containing 10 mM NaCl. The adsorbed fraction was eluted with equilibration buffer containing 0.5M NaCl

The molecular mass of Asnilase was 33,800 Da with the isoelectric point of 4.2. Enzyme specificity of Asnilase was measured by synthetic peptides GAAPLNA, STANA and SAPANA for elastase. Among these substrates, GAAPLNA was hydrolyzed most actively and the specific activity was 334.9 μmole/min/mg protein.

Asnilase is sensitive to high temperature and lost all enzymatic activity at 60℃ although 64 % activi ty still remained at 50℃ (Table 1). The enzyme activi ty of Asnilase was stable over pH range of 6-10 and highest at pH of 7.5. The enzyme activity was completely lost by the inhibitors *p*-amidinophenyl methylsulfonylfluoride (APMSF) and *N*-bromosuccinimide (NBS) at the final concentrations of 2.5 mM and *p*-chloromercuribenzoic acid (PCMB) at 1 mM when GAAPLNA was as the substrate. Ethylene glycol tetraacetic acid (EGTA) at 1 mM also showed some inhibitory effect and only 17.9 % of enzyme activity retained. However, neither dithiothreitol nor benzamidine had any inhibitory effect.

Several elastolytic proteases [8, 10, 11, 13, 14] and alkaline proteases [9, 12, 15, 16] have been isolated from *Aspergillus* species. Various extracellular proteases were also purified from *A. nidulans* and characterized [17, 18, 29-31], and among these enzymes, proteinase I and II [17] and alkaline protease [18] showed similar molecular mass, isoelectric points and stability to Asnilase. The effects of inhibitors on the enzymes derived from *A. nidulans*, *A. fumigatus* and *A. flavus* were compared in Table 1. These elastolytic enzymes were affected by serine protease inhibitors such as APMSF, PMSF and DFP. However substrate specificity was different for these elastolytic enzymes.

Table 1. Comparison of biological properties of elastolytic proteases from various *Aspergillus* species

Property	Elastolytic protease from			Alkaline protease	Proteinase I	Proteinase II
	A. nidulans	***A. fumigatus***	***A. flavus***	from ***A. nidulans***		
	This study	[11]	[14]	[18]	[17]	
Molecular mass (Da)	33,800	32,000	40,000	42,000	30,900	30,000
Isoelectric point	4.2	9.1	8.6	ND*	4.6	4.3
Enzyme specificities	GAAPLNA	azocoll hide powder azure GAAPLNA		casein gelatin	azocoll, azocasein hide powder azure *p*-nitrophenyl acetate	
Optimum pH	7.5	7.5	8.5	8.0	6.5 – 7.5	
Optimum temperature	45℃	37℃	37℃	35℃	ND	
Heat stability	50℃	50℃	50℃	50℃	45℃	
pH stability	6-10	6-10	6-10	6-10	ND	
Inhibitors	APMSF NBS PCMB EGTA	PMSF NBS DFP leupeptin	EDTA DFP leupeptin	PMSF EDTA	PMSF DFP	
Fibrinogenase activity	Aα, Bβ	Aα, Bβ, γ	Aα, Bβ, γ	ND	ND	
Collagenase Activity (digested type)	I, III, IV	I, II, III, IV	I, III, IV	ND	ND	

**ND : not determined*

Proteinase I, II and alkaline protease from *A. nidulans* strongly hydrolyzed hide powder azure and/or casein, and elastolytic proteases from *A. fumigatus* and *A. flavus* also hydrolyzed azocoll and hide powder azure, whereas Asnilase possessed no effect on these substrates. This fact indicates that Asnilase is a novel elastolytic enzyme.

3.2 Hydrolyzing effects on mammalian tissue proteins

Two important proteins commonly present in the mammalian tissues were selected for substrate specificity. One is fibrinogen that is important for blood coagulation and the other is collagen that is important for tissues. When human fibrinogen was incubated with Asnilase, Aα chain was shown to be most sensitive to proteolytic digestion and the band was completely disappeared within 15 minutes (Figure 2, left). The Bβ chain of fibrinogen was also hydrolyzed but it required longer time of incubation. It seems the γ chain of fibrinogen was resistant to hydrolysis with Asnilase. The effect of Asnilase on collagen type I, II, III, and IV were also determined and type IV collagen was significantly hydrolyzed by this enzyme (Figure 2, right). Type I and III collagen were slightly digested, whereas type II collagen was not degraded. Similar to Asnilase, elastolytic proteases from *A. fumigatus* and *A. flavus* digested collagen type I, III and IV, and in addition, the enzyme from *A. fumigatus* hydrolyzed collagen type II (Table 1).

In order to find which peptide bond can be cleaved by Asnilase, the oxidized insulin B chain was used and its proteolytic specificity was compared with other elastolytic proteases. Asnilase hydrolyzed only His(5)-Leu(6) and Glu(13)-Ala(14) bonds, however enzymes from *A. fumigatus* and *A. flavus* cleaved insulin B chain at 7 and 11 sites, respectively [14]. This indicates that the substrate specificity of Asnilase is selective and is different from other elastolytic enzymes.

Patterson et al. [1] reported that *A. fumigatus* is the most common species found in invasive aspergillosis (66 % of 261 cases) and responsible for severe illness, while the rates of detection of *A. flavus* (14 %) and *A. nidulans* (0.4 %) are relatively low. The pathogenic difference among these species possibly depends on the substrate specificity. The broad spectrum of *A. fumigatus* enzyme against various collagen types and other proteins may be responsible for the destruction of various organ and tissues.

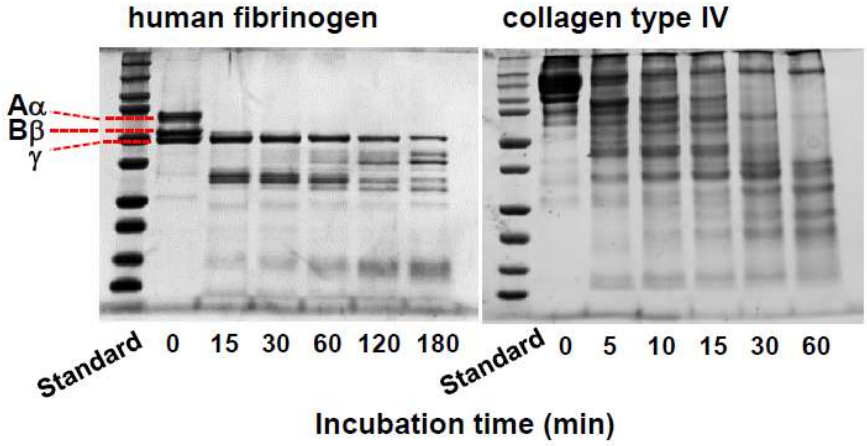

Fig. 2. Fibrinogen and collagen hydrolytic activity of elastolytic protease
Human fibrinogen (1 mg) or type IV collagen (1.35 mg) was mixed with 0.38 μg of Asnilase, respectively. Incubation was performed for indicated time intervals and the reaction mixture was analyzed by SDS-PAGE.

3.3 Cytotoxic effect on human cells

Cultured human cells (HPAEC: human pulmonary artery endothelial cells, HSAEC: human small airway epithelial cells, HBTEC: human bronchial/tracheal epithelial cells, HPAEpiC: human pulmonary alveolar epithelial cells) were employed to determine the cytotoxic activity of Asnilase (Figure 3). After

the exposure to the enzyme, the average of living cell count in each well was clearly decreased in a concentration-dependent manner, and the 50 % effective concentrations (EC_{50}) were 3.0 μg/ml for HPAEC, 2.0 μg/ml for HSAEC and 6.0 μg/ml for HPAEpiC, respectively. Endothelial cells were most sensitive to Asnilase and viable cell count drastically decreased for the concentrations of 2.0 to 4.0 μg/ml. Pulmonary alveolar epithelial cells also die in a similar manner for the concentrations of 3.0 to 10 μg/ml. The effect of Asnilase to bronchial/tracheal epithelial cells was relatively moderate, and more than 50 % of cells were alive at the concentration of 10 μg/ml.

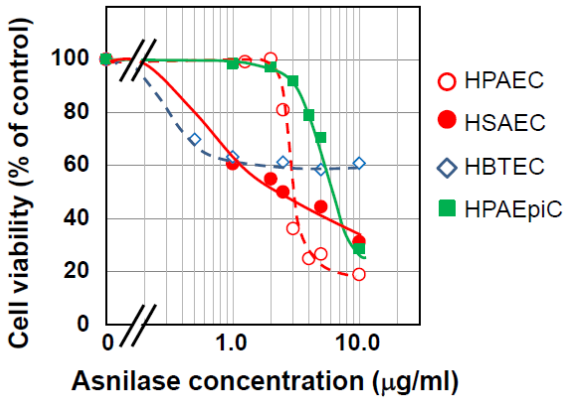

Fig. 3. Effect of Asnilase on various cultured human cells

Human cells were seeded in 96 well micro-titer plates at a density of 1.5×10^4 cells/well. Asnilase was diluted in appropriate medium, then added to the cells (n=5) at a final volume of 0.1 ml per well. Cells were incubated for 24 hours in a CO_2-incubator, and viable cell count in wells were determined by the colorimetric method. The mean values of 5 experiments versus control (cells number incubated in the absence of enzyme) were expressed as the percentage.

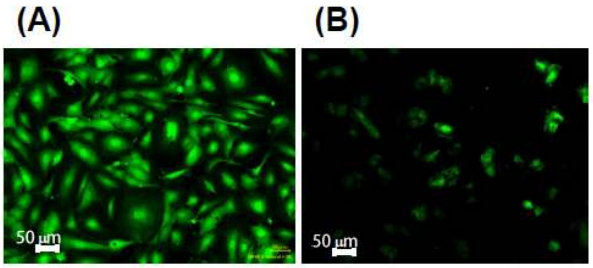

Fig. 4. Fluorescence microscopic images of HPAEC

Human pulmonary endothelial cells (HPAEC) were incubated with Asnilase (1.0 μg/mL) for 24 hours, and CellTracker™ Green CMFDA dye was employed to detect living cells.
Fluorescence microphotographs of control cells (A) and HPAEC incubated with Asnilase (B).

The adhered cells to the surface of well were stained with fluorescence dye and observed under a fluorescence microscope. The concentrations of Asnilase which did not cause cell death clearly decreased the adhesive property of the cells to the plate surface. For example, 1.0 μg/mL of Asnilase did not affect the viability of HPAEC with 24 hours exposure (Figure 3) whereas remaining HPAEC on the plate surface was clearly decreased with 1.0 μg/mL of this enzyme. As shown in Figure 4B, detachment of most of the cells was obvious and very few adhered cells remained on the plate surface after 24 hours. Time lapse observation of cells indicated that cytomorphologic changes started after 3 hours exposure and detachment of cells gradually occurs subsequently (data not shown). These phenomena are remarkable in HPAEC, but not observed clearly with HBTEC.

Reichard et al. [8] reported that Alp (extracellular serine proteinase from *A. fumigatus*) efficiently detached Vero cells from a plastic surface at concentrations of 1.0 μg/mL after 10 hours exposure. The detached Vero cells were viable and could be propagated in culture, however the effect of Asnilase on HPAEC by was irreversible. The difference between the effect of Alp and Asnilase may be due to difference in cell strain which used. From the mentioned results, Asnilase induce cytomorphologic changes of cells to decrease adhesive property at first, and the exfoliated cells subsequently lose their function and gradually reach to the death.

It may be associated with the pathogenicity of *Aspergillus* species that HPAEC and HPAEpiC show high susceptibility for elastolytic enzyme. The injury of vascular endothelial cells results in hemorrhage, and bleeding may be enhanced more by the fibrinogenolytic effects of Asnilase. Also, the destruction of alveolar cells by this elastolytic enzyme may facilitate invasion process of *Aspergillus.* The relatively weak injury characteristic for bronchial cells does not cause the large lesion, but may cause the allergic disease.

4. CONCLUSION

It is shown in this report that Asnilase, an elastolytic proteinase produced by *A. nidulans* presumably contribute to pulmonary aspergillus infection by its cytotoxicity and hydrolytic activity on proteins from biotissue.

REFERENCES

1. Patterson TF, Kirkpatrick WR, White M, Hiemenz JW, Wingard JR, Dupont B et al. Invasive aspergillosis. Disease spectrum, treatment practices, and outcomes. 2000;79, 250-60.
2. Thompson III GR, Patterson TF. Pulmonary aspergillosis. Semin Respir Crit Care Med. 2008;28(2), 103-10.
3. Kothary MH, Chase T Jr, Macmillan JD. Correlation of elastase production by some strains of *Aspergillus fumigatus* with ability to cause pulmonary invasive Aspergillosis in mice. Infect Immun. 1984;43(1), 320-5.
4. Rhodes JC, Bode RB, McCuan-Kirsch CM. Elastase production in clinical isolates of *Aspergillus.* Diagn Microbiol Infect Dis. 1988;10, 165-70.
5. Kolattukudy PE, Lee JD, Rogers LM, Zimmerman P, Ceselski S, Fox B et al. Evidence for possible involvement of an elastolytic serine protease in Aspergillosis. Infect Immun. 1993;61(6), 2357-68.
6. Mellon JE, Cotty PJ. Expression of elastinolytic activity among isolates in *Aspergillus* Section *Flavi*. Mycopathologia. 1995;131, 115-20.
7. Starcher BC. Elastin and the lung. Thorax. 1986;41, 577-85.
8. Reichard U, Büttner S, Eiffert H, Staib F, Rüchel R. Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. J Med Microbiol. 1990;33, 243-51.
9. Monod M, Togni G, Rahalison L, Frenk E. Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. J Med Microbiol. 1991;35, 23-8.
10. Frosco M, Chase T Jr, Macmillan JD. Purification and properties of the elastase from *Aspergillus fumigatus*. Infect Immun. 1992;60, 728-34.
11. Hasegawa Y, Nikai T, Yamashita R, Yoshikawa Y, Sugihara H, Ogawa K, Mizuno M. Isolation and characterization of elastolytic proteinase from *Aspergillus fumigatus*. Jpn J Med Mycol. 1995;36, 235-43.
12. Wang SL, Chen YH, Wang CL, Yen YH, Chern MK. Purification and characterization of a serine protease extracellularly produced by *Aspergillus fumigatus* in a shrimp and crab shell powder medium. Enz Microb Technol. 2005;36, 660-5.
13. Rhodes JC, Amlung TW, Miller MS. Isolation and characterization of an elastinolytic proteinase from *Aspergillus flavus*. Infect Immun. 1990;58(8), 2529–34.
14. Hasegawa Y, Nikai T, Okumura Y, Ogawa K, Sugihara H. Characterization of elastolytic proteinase from *Aspergillus flavus* : Comparison of biochemical properties to the elastolytic proteinase from *Aspergillus fumigatus*. Jpn J Med Mycol. 1997;38, 53-9.
15. Chakrabarti SK' Matsumura N, Ranu RS. Purification and characterization of an extracellular alkaline serine protease from *Aspergillus terreus* (IJIRA 6.2). Curr Microbiol. 2000;40(4), 239-44.
16. Tunga R, Shrivastava B, Banerjee R. Purification and characterization of a protease from solid

state cultures of *Aspergillus parasiticus*. Process Biochem. 2003;38(11), 1553–8.
17.Ansari H, Stevens L. Purification and Properties of Two Neutral Proteinases from *Aspergillus nidulans*. J Gen Microbiol. 1983;129, 1637-44.
18.Charles P, Devanathan V, Anbu P, Ponnuswamy MN, Kalaichelvan PT, Byung-Ki H. Purification, characterization and crystallization of an extracellular alkaline protease from *Aspergillus nidulans* HA-10. J Basic Microbiol. 2008;48, 347–52.
19.Ogawa K, Hasegawa Y, Nikai T, Sugihara H, Takagi K. Elastase from *Aspergillus fumigatus.* –Its pathogenetic role and inhibitor-. Jpn J Med Mycol. 1997;38(2), 149-53.
20.Hasegawa Y, Nikai T, Shibata Y, Komori Y, Homma M, Ogawa K, Sugihara H. The role of virulence factor of elastolytic proteinase from *Aspergillus fumigatus*. Jpn J Med Mycol. 1999;40(2), 82-91.
21.Weber K, Osborn M. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem. 1969;244(16), 4406-12.
22.Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72, 248-54.
23.Okumura Y, Matsui T, Ogawa K, Uchiya K, Nikai T. Biochemical properties and primary structure of elastase inhibitor AFUEI from Aspergillus fumigatus. J Med Microbiol. 2008;57, 803–8.
24.Murata Y, Satake M, Suzuki T. Studied on snake venoms. Distribution of proteinase activities among Japanese and Formosan snake venom. J Biochem. 1963;53, 431-7.
25.Civello DJ, Moran JB, Geren CR. Substrate specificity of a hemorrhagic proteinase from timber rattlesnake venom. Biochemistry. 1983;22, 755-62.
26.Charney J, Tomarelli RM. A colorimetric method for the determination of the proteolytic activity of duodenal juice. J Biol Chem. 1947;131, 501-5.
27.Ishiyama M, Miyazono Y, Sasamoto K, Ohkura Y, Ueno K. A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta. 1997;44(7), 1299-305.
28.Tominaga H, Ishiyama M, Ohseto F, Sasamoto K, Hamamoto T, et al. A water-soluble tetrazolium salt useful for colorimetric cell viability assay. Anal Commun. 1999;36, 47-50.
29.Peña-Montes C, González A, Castro-Ochoa D, Farrés A. Purification and biochemical characterization of a broad substrate specificity thermostable alkaline protease from *Aspergillus nidulans*. Appl Microbiol Biotechnol. 2008;78, 603-12.
30.Emri T, Szilágyi M, László K, M-Hamvas M, Pócsi I. PepJ is a new extracellular proteinase of *Aspergillus nidulans*. Folia Microbiol. 2009;54(2), 105-9.
31.Castro-Ochoa D, Peña-Montes C, Farrés A. Evaluation of strategies to improve the production of alkaline protease PrtA from *Aspergillus nidulans*. Appl Biochem Biotechnol. 2013;169, 1672-82