

## PRODUCTION OF SERINE CHYMOTRYPSIN-LIKE ELASTASE BY ASPERGILLUS FUMIGATUS STRAINS

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### SUMMARY

Thirtyfour *Aspergillus fumigatus* strains isolated from air, horse-hair, agricultural soil and human samples were screened to evaluate the production of elastase.

*Aspergillus fumigatus* strains were grown in elastin solid medium, showing a widespread elastin solubilization.

However, isolates from human and agricultural soil samples were found to be the highest elastase producers.

Then, eight out of 34 strains were grown in four different liquid media, on which we investigated total and specific proteolytic activity. Results from this experiments suggest that the elastase production is induced by the presence of elastin as a substrate and that the elastase is a chymotrypsin like enzyme.

Inhibitory profile showed that the *A. fumigatus* elastase is a serine proteinase.

### INTRODUCTION

*Aspergillus fumigatus* Fres. is a widespread thermotolerant species. It is also a pathogenic fungus able to induce a variety of lung disease in man, i.e. allergic aspergillosis fungus balls, and invasive aspergillosis. The later disease is particularly severe in immunocompromised patients. (2,4). It is known that members of the genus *Aspergillus* may produce a number of proteolytic enzymes (3,10) and it has been suggested that the production of such enzymes may be associated with virulence of the fungal strain.

There is an increasing body of evidence sug-

### RESUMEN

[Producción de una elastina semejante a serina-quimiotripsina por cepas de *Aspergillus fumigatus*]

Treinta y cuatro cepas de *Aspergillus fumigatus* aisladas del aire, crín de caballo, suelo agrícola y del hombre, fueron examinadas con el fin de evaluar la producción de elastasa.

Las cepas de *Aspergillus fumigatus* fueron cultivadas en un medio sólido con elastina, apreciándose en ella su amplia solubilización por la acción del hongo.

Los aislamientos fúngicos provenientes de muestras aisladas del hombre y de suelos agrícolas fueron detectados como los más altos productores de elastasa.

Ocho de las 34 cepas fueron desarrolladas en 4 diferentes medios líquidos en las cuales se investigó la actividad proteolítica total y específica. Los resultados de este experimento sugieren que la producción de elastasa es inducida por la presencia de elastina como sustrato y que la primera es una enzima semejante a la quimiotripsina.

El perfil inhibitorio comprobó que la elastina de *A. fumigatus*, es una serina-proteinasa.

gesting that *A. fumigatus* may produce an extracellular proteinase able to degrade elastin (4,9,12,5) and that such an elastase is related to the ability of *A. fumigatus* to induce invasive aspergillosis in animal models (5).

In this contribution, we examined *in vitro* elastase production by 34 *A. fumigatus* strains isolated from different sources (soil, plant, human and horse-hair) and we achieved a partial characterization of this enzyme in members of this species group.

The reasons why *A.fumigatus* is more invasive than the other species of the genus remain as unexplained: we will present and discuss some of our own experimental data concerning the elastase production from these strains in relation to their ecology and to pathogenic activity.

## MATERIALS AND METHODS

### Origin of *A.fumigatus* strains

The majority of the 34 *A.fumigatus* strains used in this study were freshly isolated from various sources such as air (7 strains), horse-hair (2 strains), human sputum or bronchoaspirate (10 strains), and 15 strains isolated from agricultural soils (wheat-corn, ricefield soil) and grapevine leaf.

### Elastase production in solid medium

The strains were cultured (25°C) on Czapek-agar (Biolife) until sufficient growth had occurred. The cultures were used to provide the inoculum required; spores were collected in sterile 0.1 % Tween 80 (polyoxyethylene Sorbitan monooleate) and a drop of spore suspension at the concentration of 10<sup>7</sup>/ml was inoculated, in a central spot, onto agar medium (25 ml in Petri dishes) containing elastin, according to Kothary et al. (5).

Cultures were incubated at 37°C for 7 days, elastase activity was detected visually as a clearing zone around and beneath the colony.

### Growth in liquid media

Eight *A.fumigatus* strains showing to be strong extracellular elastase producers, were tested for the production of elastase in the following liquid media: Czapek-Dox broth (Biolife); YCB + peptone [0.2 % YCB, 0.2 % peptone (Difco), in 0.05 M borate buffer pH 7.6]; elastin [0.2 % soluble bovine neck ligament elastin (Elastin products co.), [0.2% YCB in 0.05 M borate buffer, pH 7.6]; and elastin + albumin [elastin medium + 0.05% bovine albumin (Sigma)], according to Rhodes et al. (14).

A drop of the suspension obtained as previously described was inoculated into 50 ml of each growth medium in a 250 ml. baffled flask and incubated on a shaker, 100 rpm) at 37°C for 72 h.

The culture broth was filtered on paper (Whatman n.4) and finally concentrated approximately 33 fold by ultrafiltration (YM 10 membranes, Amicon). Samples were divided in aliquotes and kept at -20°C until needed.

A sample of each medium without fungus in-

oculation, was processed in the same fashion and used as control.

### Specific proteolytic activity

To this aim we used the azocasein assay according to the procedure of Barrett (1). Briefly, 250 ul of each sample were incubated with 0.5% azocasein (Sigma) in TRISHCL 0.2M, pH 7.4 at 37°C.

The reaction was stopped after 15 minutes with 200 ul of acetic acid 1N and the absorbance read at 440 nm (PYE Unicam). All samples were run in duplicate with appropriate blanks.

Values were expressed as mg. azocasein degraded over time.

### Hydrolysis of synthetic peptide substrates

To this aim 20 ul of each sample were incubated for 1 h. at 37°C in phosphate buffer 0.2M pH 7.4, with the following substrates: N-Succinyl-(Ala)<sub>3</sub>-p-Nitroanilide 0.1 mM (SApNa; Sigma); N-methoxysuccinyl-(Ala)<sub>2</sub>-Pro-Val-p-Nitroanilide 0.1 mM [Me (0)AAPVpNA; Sigma]; Succinyl-(Ala)<sub>2</sub>-Pro-phe-p-Nitroanilide 0,1 mM (SAAPPhep Na; Sigma).

At the end of incubation the reaction was stopped and the absorbance read at 410 nm. The values were expressed as nMoles of substrate hydrolyzed over time.

### Protein assay

Protein concentration was measured according to Lowry and coworkers (7).

### Inhibitory study

In order to assign the hydrolytic activity on the synthetic peptide substrates to the class of metallo or serine-proteinase, some samples were preincubated with the metalloproteinase inhibitor EDTA 10 mM (Merck) and the serine-proteinase inhibitor phenylmethylsulfonyl fluoride 10 mM (PMSF; Sigma) for 30 minutes. There after we proceeded as above described.

### Insoluble elastin degradation

To this aim we used the method described by Shotton (15). Briefly, 7 ml of borate buffer 0.02M, pH 8,8 containing elastin Congo red (Sigma) at concentration of 1 mg/ml, were added to 2 ml of the sample (strain N° 13 grown for 72 h on liquid elastin medium). The mixture was incubated at 37°C. on shaker and every 15 minutes an aliquot was drawn, centrifuged and the absorbance of the supernate read at 495 nm. In separate tube, elastin Congo red was incubated with 40 ug of porcine

Table 1. Elastinolytic activity on solid medium of different *A.fumigatus* strains.

<i>A.fumigatus</i> strain no.	Source	O of growth area	O of elastin solubilization area (mm)
1	air	0	0
2		0	0
3		0	0
4		0	0
5		0	0
6		0	0
7		9	9
8	horse	27	28
9		15	19
10	human	26	20
11		44	44
12		38	38
13		10	20
14		35	35
15		40	35
16		11	20
17		18	24
18		19	25
19		40	40
20	wheat field	0	0
21		0	0
22		95	65
23		0	0
24		85	85
25		39	39
26	corn field	95	95
27		95	95
28		85	85
29		25	25
30	rice field	35	35
31		25	25
32		28	28
33		95	95
34	grapevine leaf	75	75

Table 2. Activity of different *A.fumigatus* strains on Azocasein and synthetic peptide substrate

<i>A.fumigatus</i> strain no. & source	Medium m	Azocasein assay #	Synthetic peptide* assay	Total prote
8 (horse)	Cz	na	0.048	5.242
	YCB/pep	0.007	0.007	21.73
	ela	0.161(0.001)	0.207(0.001)	115.94
	ela/alb	0.186(0.0002)	0.223(0.0002)	862.752
9 (horse)	Cz	na	0.063	1.759
	YCB/pep	0.671	0.870	6.875
	ela	1.184(0.016)	0.973(0.013)	72.650
	ela/alb	0.916(0.002)	0.455(0.001)	385.440
10(human)	Cz	na	0.005	2.04
	YCB/pep	0.223	0.289	3.109
	ela	0.889(0.019)	0.924(0.019)	46.34
	ela/alb	1.154(0.001)	0.721(0.001)	586.974
13(human)	Cz	na	0.010	3.779
	YCB/pep	0.144	0.380	10.450
	ela	0.612(0.005)	1.083(0.009)	108.372
	ela/alb	1.937(0.001)	1.395(0.0007)	1,925.00
16(human)	Cz	na	0.0033	0.33
	YCB/pep	0.063	0.054	4.884
	ela	0.711(0.007)	0.980(0.009)	98.761
	ela/alb	1.846(0.001)	1.546(0.0009)	1,001.123
24 (wheat field)	Cz	na	0.006	3.82
	YCB/pep	na	na	8.31
	ela	0.314(0.005)	0.720(0.013)	54.1
	ela/alb	0.722(0.0005)	1.092(0.0008)	1,349.56
31 (rice field)	Cz	na	0.016	3.894
	YCB/pep	na	0.003	2.904
	ela	0.088	0.308	1,488.96
	ela/alb	0.065	0.034	2,530.50
34 (grapevine leaf)	Cz	na	0.016	2.70
	YCB/pep	na	0.027	7.896
	ela	0.957(0.017)	0.789(0.014)	55.75
	ela/alb	1.079(0.0013)	0.851(0.001)	820.10

m Cz=Czapek; YCB/pep= yeast carbon base+peptone; ela = elastin; ela/alb = elastin+ albumin; # mg of azocasein degraded in 15 min. In brackets, value normalized per mg of proteins.\* nMoles of SAAPPhepNA hydrolyzed in 1h. In brackets, value normalized per mg of proteins. \$ mg. dl-1. na= no activity

pancreatic elastase (PPE; Sigma, type IV) and the experiment then proceeded as previously described. Values were expressed as changes of absorbance over time.

## RESULTS

Table 1 shows the growth and the elastolytic activity of *A. fumigatus* strains in solid medium. Only one of seven strains isolated from air showed moderate growth and elastinolysis on this medium, whereas almost all the strains isolated from the other sources (human, horse-hair, cultivated soil) were able to grow and to solubilize insoluble elastin.

In some cases the growth and elastinolysis had the same degree (fig. 1A), whereas in the other cases the diameter of elastinolysis exceeded the diameter of growth (fig. 1B). Strains of *A. fumigatus*, isolated from agricultural soils were the high elastase producers.

As it may be ascertained from the inspection of table 2, none of eight strains, selected on the basis of previous results and grown in Czapek liquid medium, revealed a proteolytic activity, as detected by azocasein assay. In five out of the eight strains, this activity was fairly enhanced when the liquid medium was the YCB+peptone. A dramatic increase in the proteolytic activity was determined by the growth of strains in elastin medium. In most of the cases the addition of albumin to elastin medium induced only a moderate further increase of proteolytic activity.

With regard to the hydrolysis of the three synthetic peptide substrates used, the eight strains did not react either with SApNa or Me(0) AAPVpNa substrates (data not shown).

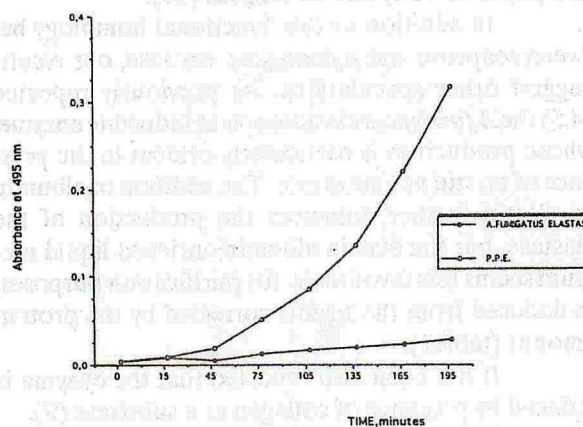
By contrast, samples strongly hydrolyzed the SAAPPhepNa substrate, thus suggesting a preferential cleavage of a substrate with phenylalanine residue in P<sub>1</sub> position. The results of the hydrolysis of SAAPPhepNa substrate by the *A. fumigatus* strains in different liquid media, strictly paralleled those obtained in the azocasein assay.

Concerning the inhibitory profile of the hydrolytic activity, we found that the cleavage of the SAAPPhepNa substrate was fully abolished by PMSF, whereas it was unaffected by EDTA (data not shown). Therefore we may assign this activity to the class of serine proteinase.

Finally the elastin Congo red degradation assay allowed us to establish that the elastase produced by *A. fumigatus* in elastin medium has a moderate elastolytic capacity, when compared to the elasti-

nolytic capacity of PPE (fig. 2). In fact the elastolytic capacity of *A. fumigatus* was 7,6 % of the PPE elastolytic capacity.

Fig 2. Elastin -congo red solubilization



## DISCUSSION

Our data show that the most of the *A. fumigatus* strains tested are able to produce an elastase when cultured in agar solid medium containing insoluble elastin, with the exception of most of the strains which were isolated from air samples. This finding is consistent with the putative virulence role of the elastase (5) in the pathogenesis of aspergillosis in which *A. fumigatus* is the primary agent. However differences in the degree of elastase production were found within strains isolated from the same source.

Several speculations may be drawn from the liquid media results. First of all, the preference of the elastase for a synthetic peptide substrate containing a Phe residue in P<sub>1</sub> position suggests a chymotrypsin-like activity (17). Our results are consistent with those previously reported by Reichard and coworkers (12). The lack of activity of *A. fumigatus* elastase with substrates containing Val or Ala residues in P<sub>1</sub> position are consistent with previously results obtained by some of us (8) on alkaline proteinase (called "seaprose") produced by *A. melleus* Yukawa, a nonpathogenic mould belonging to *A. alutaceus* (formerly *A. ochraceus*) group (6). The inhibitory profile shows that the *A. fumigatus* elastase is a serine-proteinase, whereas the ability to degrade insoluble elastin, as suggested by the elastin-congo red assay, is rather weak when compared with that showed by porcine pancreatic elastase. Both of these characteristics are shared by seaprose.

Since *A. ochraceus* has been reported as human pathogenic only occasionally (11), the homology between *A. fumigatus* elastase and seaprose, the *A.*

*melleus* elastase, actually raises concern on the role of *A. fumigatus* elastase as a virulence factor in human infection, at least as a main factor from this point of view (4-5). This concern recently found a confirmation in a paper of Tony and colleagues (16).

In addition to this functional homology between seaprose and *A. fumigatus* elastase, our results suggest other speculations. As previously reported (4,5) the *A. fumigatus* elastase acts as inducible enzyme, whose production is particularly evident in the presence of elastin as a substrate. The addition of albumin to elastin further enhances the production of the elastase, but the elastin albumin enriched liquid medium seems less favourable for purification purposes, as deduced from the results corrected by the protein amount (table 2).

It has been also reported that the enzyme is induced by presence of collagen as a substrate (9).

Secondly, the characterization of the *A. fumigatus* elastase by different authors (9) yielded somewhat contrasting results. For instance, Monod and coworkers

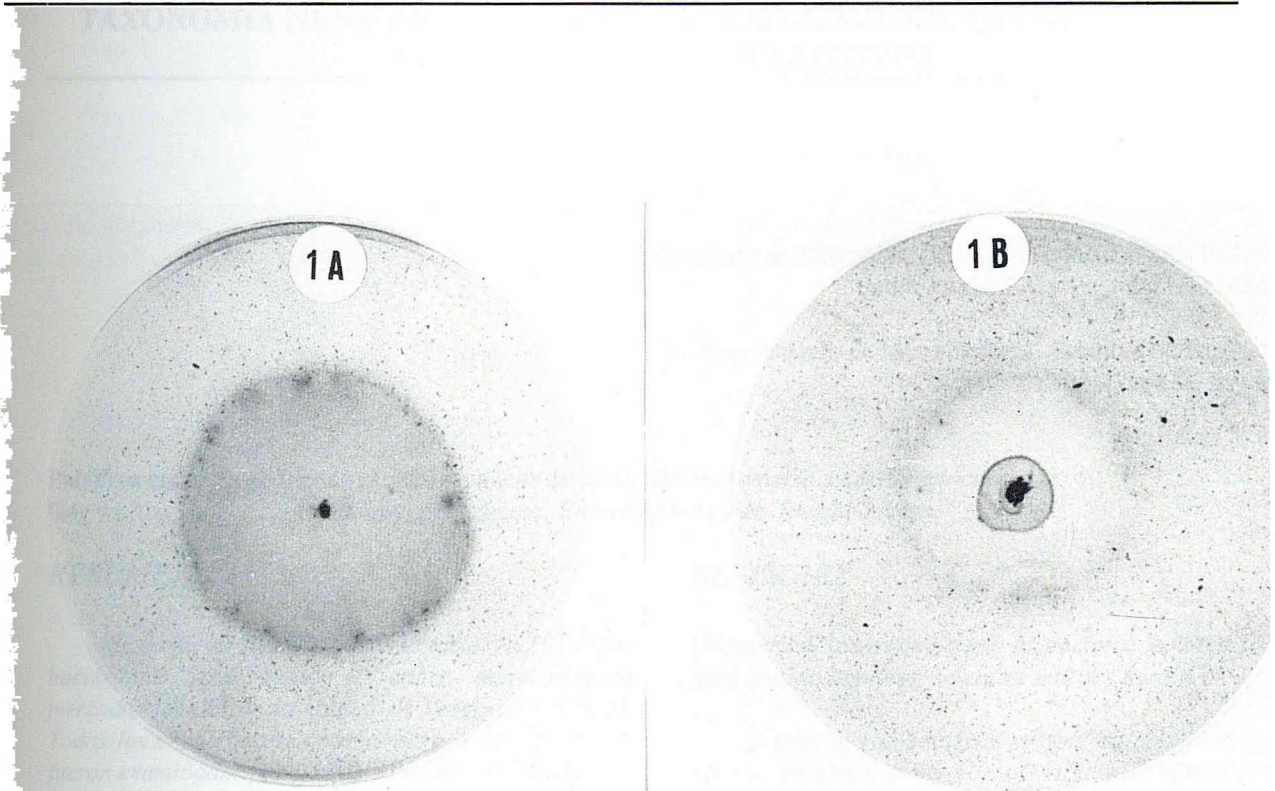
(9,12,4) reported a 100 % inhibition by PMSF, but no inhibition by either EDTA or TPCK (the latter being a chymotrypsin inhibitor). By contrast, Reichard and coworkers (12) reported a partial inhibition by TPCK, whereas Frosco and coworkers (4) described a 100 % inhibition by 5 mM EDTA, thus suggesting that *A. fumigatus* elastase is a serine proteinase stabilized by ion metals. These discrepancies may imply that *A. fumigatus* could produce more than a single type of elastase.

In our hands, the good agreement between activity of *A. fumigatus* liquid media on specific synthetic peptide substrate and total proteolytic activity, i.e. azocasein assay, would suggest that the serine-chymotrypsin like elastase is the major elastase produced by *A. fumigatus* under experimental conditions.

Our results in agreement with Rhodes et al. (13) demonstrated elastinolytic activity in all strains of *A. fumigatus* isolated from clinical cases, but also a strong elastinolytic activity in saprophytic strains isolated from agricultural soils.

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**Legends to figures.**

Fig.1A.- *A.fumigatus* growth in elastin solid medium. Same degree of growth and elastinolysis (1A). The elastinolysis exceeded the growth (1B).

Fig. 2B.- Elastin congo red degradation by *A.fumigatus* and porcine pancreatic elastase (P.P.E.)