Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*

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Summary. *Aspergillus fumigatus* secreted an inducible alkaline protease (AlPase) when cultivated in the presence of collagen (200 µg/ml) as sole nitrogen and carbon source. Proteolytic activity was maximum at pH 9.0 with azocollagen as substrate. The enzyme, which was the major protein found in the supernate of a liquid culture, was purified by ammonium sulphate precipitation and gel filtration. The M_r was determined to be 33 Kda by gel filtration and sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The isoelectric point was estimated to be pH 8.2. Divalent cations strongly inhibited enzyme activity, whereas non-ionic detergents and reducing agents had no effect. *A. fumigatus* AlPase was totally inhibited by phenylmethanesulphonyl fluoride, antipain, chymostatin and α -2-macroglobulin. *A. fumigatus* AlPase is closely related to the *A. oryzae* AlPase, a serine protease of the subtilisin family, as attested by the antigen pattern seen by immunoblotting. The high collagenic activity and the ability of *A. fumigatus* AlPase to digest elastin could play a role in the invasion of the tissues by the fungus.

Introduction

Aspergillus fumigatus is a highly pathogenic fungus which causes broncho-pulmonary allergies, aspergillomas (fungus balls) and invasive aspergillosis.¹ Reports of the incidence of invasive aspergillosis have increased in recent years, particularly in neutropenic patients. Consequently, interest in the pathogenic mechanisms of this fungus has increased.

Recent data suggest that the virulence of *A. fumigatus* is associated with the production of extracellular proteases. In mice, a correlation was established between elastase production by strains of *A. fumigatus* and the ability to cause invasive pulmonary aspergillosis.² Elsewhere, it was shown that the proteolytic activity in the mouse brain increased in proportion to the growth of the fungus.³ Species of the genus *Aspergillus* produce many different extracellular enzymes.⁴ A 37-Kda extracellular acid protease from *A. fumigatus* has been purified and characterised.^{5,6} In contrast, few data are available on neutral and alkaline exoproteases of this species.^{7,8} We examined clinical strains of *A. fumigatus* for production of an alkaline protease (AlPase) with a high collagenolytic activity and ability to digest elastin which could play a role in the invasion of lung tissue by the fungus, and compared the enzyme with the alkaline protease of *A. oryzae*, a species of *Aspergillus* used in the manufacturing process of fermented food in the Orient.⁴

Materials and methods

Materials

Malt agar was purchased from Oxoid (Basingstoke, Bucks) and soluble collagen from Serva (Heidelberg, Germany). Azocollagen, elastin-Congo red, casein, Tris base (Tris), ammonium sulphate, Triton X100, Tween 80, 2-mercaptoethanol, 1,4 dithiothreitol (DTT) and Freund's adjuvant, complete and incomplete, were from Sigma (St Louis, MS, USA). Polyacrylamide P60 gel, low-mol.-wt protein standards, and all reagents used for immunoblotting—nitrocellulose membranes, gelatin, Tween 20, alkaline phosphatase-conjugated goat anti-rabbit IgG, nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)—were from BioRad. DEAE Sephadex, ampholytes of pH range 3.5–9.5 and isoelectric focusing standards were from Pharmacia (Uppsala, Sweden). The protein inhibitors (4-amidinophenyl)-methanesulphonyl fluoride (APMSF), antipain di-hydrochloride, aprotinin, bestatin, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride (TLCK), L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (TPCK), chymostatin, E 64, leupeptin, α -2-macroglobulin, pepstatin, phenylmethylsulphonyl fluoride (PMSF), phosphoramidon and soybean trypsin inhibitor (SBTI) were obtained from Boehringer (Mannheim, Germany).

Strains and growth conditions

Ten strains of *A. fumigatus* were isolated from infected lung tissue and sputum of different patients

at the Centre Hospitalier Universitaire Vaudois. The strains were maintained on malt agar slants at 4°C. For inoculation, conidia from *A. fumigatus* were produced in bulk by growth on malt agar at 30°C for 5–10 days. For enzyme production, the strains were grown in liquid medium containing soluble collagen 0.2%, w/v as sole nitrogen and carbon source. The medium was sterilised by autoclaving at 120°C for 15 min. Flasks containing 500 ml of medium were seeded with $c. 10^7$ conidia and incubated at 30°C on an orbital shaker at 200 rpm for 70 h.

Enzyme purification

The culture supernate was separated from the mycelium by filtration through paper tissue. Ammonium sulphate was dissolved at room temperature in the supernate to 90% saturation. From this point, all steps of enzyme purification were done at 4°C. The precipitated proteins were collected by centrifugation and resuspended in 1/150th of the original volume of distilled water. Insoluble material was removed by centrifugation at 5000 *g* for 5 min, and the supernate was dialysed against 20 mM Tris-HCl, pH 8.0, for 4 h. After reduction of the volume to 5 ml by ultrafiltration, (Ultracent-30 system, BioRad), the enzyme solution was chromatographed on a column of polyacrylamide P 60 gel in 20 mM Tris-HCl buffer, pH 8.0.

Purification of *A. oryzae* protease

Crude powder (10 mg) of commercially available *A. oryzae* AlPase was resuspended in 5 ml of 20 mM Tris-HCl, pH 7.0, with 0.5 ml of DEAE Sephadex (Pharmacia) pre-swollen in the same buffer. After sedimentation of the gel, the supernate was chromatographed through a polyacrylamide P 60 gel column as described for *A. fumigatus* AlPase.

Protein concentration

Protein concentrations were measured by the method of Bradford⁹ with a BioRad dye reagent (no. 500-0006) and casein as protein standard.

Proteolytic assays

The AlPase activity was measured on azocollagen at different pH values in citrate buffer (50 mM, pH 4–7), in Tris-HCl buffer (50 mM, pH 6–10) and in glycine-NaOH buffer (100 mM, pH 8–12). Activities measured at the same pH with different buffers were identical. From a suitably diluted protease solution in buffer at 30°C, 1 ml was added to 10 mg of substrate. After incubation for 10 min with continuous shaking at 30°C, the reaction was stopped by removing the insoluble collagen by centrifugation. The absorbance at 520 nm of the coloured supernate was measured. After digestion, 1 mg of substrate gave an absorbance value of 0.60. For practical purposes, one unit of

enzyme activity was defined as that producing an increase in absorbance of 0.001 minute at pH 9.0, which corresponded to the digestion of 1.65 µg of azocollagen.

Activity against elastin was measured with elastin Congo red as substrate at a concentration of 10 mg/ml in 50 mM Tris-HCl, pH 6–10. The reaction was stopped by centrifugation. The absorbance of the orange supernate was measured at 495 nm; 1 mg of substrate, when digested, gave an absorbance value of 0.42.

Activity against heat-denatured casein was measured at a concentration of 5 mg/ml in 50 mM Tris-HCl, pH 6–10. After precipitation by trichloroacetic acid (4% final concentration) and centrifugation, soluble peptides remaining in the supernate were measured by the method of Lowry *et al.*,¹⁰ 1 mg of substrate, when digested, gave an absorbance value of 1.71.

Heat stability

A. fumigatus AlPase, 1 µg in 1 ml of 50 mM Tris-HCl buffer (pH 9), was pre-incubated in a sealed tube for 10 min at 4, 30, 40, 50, 60 and 80°C before the assay. Enzymic activity was measured as described above after adding the mixture to 10 mg of azocollagen.

Effect of detergents, organic solvent, reducing agents and divalent cations

The actions of detergents [sodium dodecyl sulphate (SDS), Triton × 100 and Tween 80] as well as organic solvents [dimethyl sulphoxide (DMSO) and ethanol], reducing agents (2-mercaptoethanol and DTT) and divalent cations on *A. fumigatus* AlPase were tested. The enzyme, 1 µg in 1 ml of 50 mM Tris-HCl buffer (pH 9), was pre-incubated with each reagent or the divalent cations for 10 min at 30°C. Enzymic activity was assayed as described above by adding the enzyme mixture to 10 µg of azocollagen. For each cation assay, an appropriate blank containing buffer, substrate and cation was treated identically. Absorbance of digested azocollagen in the enzymic assay was measured against the corresponding blank. An appropriate control containing enzyme and substrate only was assayed simultaneously.

Effects of proteinase inhibitors

The inhibitors APMSF, iodoacetamide, EDTA, antipain, leupeptin, chymostatin, phosphoramidon, E64, SBTI, α-2-macroglobulin and aprotinin were dissolved in water and PMSF, pepstatin, TLCK and TPCK were dissolved in ethanol. Either 10 or 50 µg of inhibitor was added to 1 µg of enzyme in 1 ml of 2 mM Tris-HCl buffer, pH 6.5. The mixture was incubated for 20 min at 30°C and assays were performed after adding the mixture to 10 mg of azocollagen with 50 µl of 1M Tris-HCl, pH 9. Pre-incubation in the presence of inhibitor was done at

pH 6.5 because some inhibitors were unstable at pH > 7.5. An appropriate control without inhibitor was assayed simultaneously.

Electrophoresis

Protein extracts were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli¹¹ with a separation gel of polyacrylamide 9%.

Iso-electric points

The iso-electric point was determined with a Multiphor II electrophoresis system (Pharmacia). The horizontal, non-denaturing gel contained polyacrylamide 4%, glycerol 15% and ampholytes 2.4% in the pH range 3.5–9.5. After loading the protein samples, the gel was subjected to 1 w/cm of gel length during 50 min at 4°C in an atmosphere of N₂. The iso-electric point of the purified proteins was determined by co-migration with iso-electric focusing standards (pH 3.5–9.3).

Preparation of anti-ALPase immune sera

New Zealand White rabbits were given 300 µg of purified ALPase in 0.5 ml of phosphate-buffered saline (PBS) emulsified with an equal volume of Freund's complete adjuvant by intradermal injection in the back. After 3 weeks, a booster dose of 30 µg of ALPase emulsified in Freund's incomplete adjuvant was given to increase the anti-ALPase titres. Sera were separated from the clotted blood samples.

Immunoblotting

The proteins were transferred passively on to a nitrocellulose membrane by pressing (1 lb/100 cm²) the gel between two membranes of nitrocellulose overnight in PBS buffer. About 50% of the total proteins were transferred on to each of the membranes. Protein binding sites were blocked by incubation in gelatin 3% in Tris-buffered saline solution (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5) at 22°C during 1 h. The nitrocellulose membrane was then washed with gentle agitation in TTBS (TBS + Tween 20 0.05%),

and subsequently incubated overnight with gentle agitation at 22°C with the rabbit immune antiserum diluted 1 in 1000 in TTBS. After washing twice in TTBS, the nitrocellulose membrane was incubated for 2 h at 22°C with alkaline phosphatase-conjugated goat anti-rabbit IgG at a 1 in 3000 dilution in TTBS. After washing twice with TTBS and once with TBS, the membrane was incubated for 5 min at 22°C in 50 ml of alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂) containing a mixture of 200 µl of NBT (75 mg/ml in 70% N. N-dimethylformamide) and 150 µl of BCIP (50 mg/ml in 100% N. N-dimethylformamide). The reaction was stopped by washing the membrane in water.

Results

Purification and properties of *A. fumigatus* alkaline protease

All 10 strains of *A. fumigatus* examined showed comparable proteolytic activities in collagen medium. For all strains, proteolytic activity was maximum at pH 9.0 with azocollagen as substrate. After growth for 72 h the concentration of protein in the supernate had fallen from 200 µg/ml to 6 µg/ml. No activity was detected when the fungus was grown in Sabouraud liquid medium.

Protease purification and characterisation was performed mainly with one strain (CHUV 192-88). The purification steps and yields of a typical experiment are summarised in table I. A single peak of proteolytic activity was obtained after P 60 gel chromatography. This peak corresponded to the major peak of protein eluted from the gel column. The purified enzyme migrated as a major protein band in SDS-PAGE (fig. 1, track 1). The estimated M_r of the enzyme of 33 Kda corresponded to the M_r of the protease obtained by P 60 gel chromatography. The enzyme was the major protein secreted by the fungus as shown by the protein profile of the 90% saturated ammonium sulphate precipitate of the culture supernate (fig. 1, track 3). In view of this, the overall enrichment expected after purification could be only small (table I). Although ion exchange chromatography was not necessary for its purification, *A. fumigatus* ALPase could be bound at pH 8.7 to QAE Sephadex

Table I. Purification of the extracellular alkaline protease of *A. fumigatus*

Purification step	Total volume (ml)	Total protein (µg)	Total enzyme activity (units)	Yield (%)	Specific activity (units/µg)	Purification
Culture supernate	1000	6600	98.4 × 10 ³	100	14.9	1.0
Ammonium sulphate precipitation	6	1728	59.3 × 10 ³	60.2	34.3	2.3
P60 gel filtration	14	526	42.6 × 10 ³	43.1	80.7	5.4

Fig. 1. SDS-PAGE (9% gels) of AlPase preparations (2.5 µg) of *A. fumigatus* (track F) and *A. oryzae* (track O) stained with Coomassie Brilliant Blue R-250. M_r markers: phosphorylase B, 97.4 Kda; bovine serum albumin, 66.2 Kda; ovalbumin, 42.6 Kda; bovine carbonic anhydrase, 31.0 Kda; soybean trypsin inhibitor, 21.5 Kda. Tracks 1, 2, 4 and 5: purified *A. fumigatus* and *A. oryzae* AlPases; 3: protein profile of the 90% saturated ammonium sulphate of the culture supernate. In tracks 3, 4 and 5 all steps of preparation of the samples were done in the presence of PMSF 50 µg/ml.

(Pharmacia) equilibrated with 50 mM Tris HCl, and eluted with 50 mM Tris HCl, pH 8.3. These values are consistent with the iso-electric point estimated to be pH 8.2 by iso-electric focusing.

The purified AlPase was stable for at least 2 weeks at 4°C and for 6 months at -20°C in 50 mM Tris-HCl buffer. A 10-min pre-treatment at 60°C inactivated the enzyme whereas the same treatment at 50°C had no effect. The enzyme showed a marked peak of activity at pH 9.0 and was inactive below pH 5.0 and above pH 11.0 (fig. 2). Interestingly, appreciable activity was observable on elastin-Congo red, whereas this substrate was completely resistant to trypsin (table II).

Non-ionic detergents such as Triton ×100 and Tween 80 at a concentration of 1% had no effect on AlPase activity. SDS at a concentration of 0.1% had no effect, but a decrease in activity of 60% was observed at a concentration of 1%. Neither was any effect on AlPase activity observed in the presence of organic solvents such as DMSO and ethanol, nor with

Fig. 2. Effect of pH on specific activity of *A. fumigatus* (A) and *A. oryzae* (B) AlPases, with azocollagen as substrate.

reducing agents such as 2-mercaptoethanol and DTT (table III).

A. fumigatus AlPase was very sensitive to divalent cations. Reduced activity was observed in presence of 1 mM of the eight cations tested. Concentrations of 10 mM of Mg⁺⁺, Cu⁺⁺ or Ca⁺⁺ caused inhibitions of 53, 60 and 68% respectively, whereas the enzyme was completely inactivated by 10 mM Mn⁺⁺, Zn⁺⁺, Co⁺⁺, Pb⁺⁺ or Hg⁺⁺. In contrast, a 10 mM concentration of monovalent cations such as Na⁺ or K⁺ had no effect on AlPase activity.

Different protease inhibitors were tested on *A. fumigatus* AlPase activity. The enzyme was totally inactivated by PMSF, antipain and chymostatin 10 µg/ml, and by α-2-macroglobulin 50 µg/ml. Leupeptin inhibited the enzymatic activity by 43–52% at a concentration of 10–50 µg/ml. The enzyme was not inhibited by APMSF, iodoacetamide, EDTA, pepstatin, phosphoramidon, E64, SBTI, aprotinin, TLCK or TPCK (table III).

The proteins in the culture supernates of the nine other strains of *A. fumigatus* were concentrated by 90% ammonium sulphate. The nine strains secreted a protein of identical M_r (33 Kda) that co-migrated on SDS-PAGE with the purified AlPase of strain CHUV 192–88.

Table II. Activities of *A. fumigatus* and *A. oryzae* AlPases and trypsin on different substrates at pH 7.4 and respective pH optima

Substrate	Enzyme activity* of				
	<i>A. fumigatus</i> AlPase		<i>A. oryzae</i> AlPase		Trypsin
	pH 7.4	pH optimum 9.0	pH 7.4	pH optimum 8.0	pH optimum 7.4
Azocollagen	5.01	8.80	4.04	4.75	7.58
Elastin congo red	0.073	0.130	0.026	0.057	ND
Casein	1.17	1.21	1.30	1.41	0.43

ND, not detected.

* Amount of substrate (mg) digested by 1 µg of enzyme at 30°C in 1 h.

Table III. Summary of properties of *A. fumigatus* AlPase

Molecular weight	33 000	
pH optimum	9.0	
Iso-electric point	8.2	
Heat stability	50°C for 10 min	
Effects of detergents and organic solvents		
SDS	0.1-1.0%	0-60% inhibition
Tween 80	0.1-1.0%	no effect
Triton × 100	0.1-1.0%	no effect
Ethanol	1.0-5.0%	no effect
DMSO	1.0-5.0%	no effect
Effect of reducing agents		
2-mercaptoethanol	0.1-1.0%	no effect
DTT	0.1-1.0%	no effect
Effect of divalent metal chloride		
Mg	1-10 mM	15-53% inhibition
Cu	1-10 mM	17-68% inhibition
Ca	1-10 mM	27-60% inhibition
Mn	1-10 mM	21-100% inhibition
Zn	1-10 mM	37-100% inhibition
Co	1-10 mM	72-100% inhibition
Pb	1-10 mM	75-100% inhibition
Hg	1-10 mM	94-100% inhibition
Effect of inhibitors		
Class specific		
PMSF	10-50 µg/ml	100% inhibition
APMSF	10-50 µg/ml	no effect
Iodoacetamide	10-50 µg/ml	no effect
EDTA	10-50 mM	no effect
Microbial origin		
antipain	10-50 µg/ml	100% inhibition
leupeptin	10-50 µg/ml	43-52% inhibition
chymostatin	10-50 µg/ml	100% inhibition
pepstatin	10-50 µg/ml	no effect
phosphoramidon	10-50 µg/ml	no effect
E 64	1 mg/ml	no effect
High molecular weight		
SBTI	10-50 µg/ml	no effect
α 2-macroglobulin	10-50 µg/ml	59-100% inhibition
aprotinin	10-50 µg/ml	no effect
Others		
TLCK	10-50 µg/ml	no effect
TPCK	10-50 µg/ml	no effect

Relatedness of *A. fumigatus* and *A. oryzae* AlPases

Extracellular alkaline proteases are produced by species of the *flavus-oryzae* group of *Aspergillus*. We compared the *A. fumigatus* AlPase with the *A. oryzae* AlPase commonly used in the manufacturing process of fermented food. Commercially available *A. oryzae* AlPase was purified as described. The M_r of the protein was 34 Kda, barely 1 Kda higher than the M_r of *A. fumigatus* protease on a SDS-PAGE gel (fig. 1, tracks 1 and 2) which corresponds to that reported by Tatsumi *et al.*¹² *A. oryzae* AlPase differed from *A. fumigatus* AlPase in having a much lower iso-electric point (pH 6.5) and a lower optimum pH for azocollagen digestion (fig. 2). Both enzymes digested casein with about the same efficiency, but, interestingly, the specific activity of *A. oryzae* protease against collagen or elastin was twofold lower than that of *A. fumigatus* at their respective optimal pH values (table II). Like *A. fumigatus* AlPase, *A. oryzae* AlPase

was inhibited by PMSF, antipain and chymostatin, but not by APMSF, iodoacetamide, EDTA, pepstatin, phosphoramidon, E64, SBTI, aprotinin, TLCK or TPCK. On the other hand, a concentration of α-2-macroglobulin of 50 µg/ml, which totally inhibited *A. fumigatus* AlPase, caused only 12% inhibition of *A. oryzae* AlPase.

Immunoblotting was performed with an equal amount (50 ng) of each enzyme transferred on to nitrocellulose membranes. Both proteins were detected with the same intensity by *A. fumigatus* AlPase antiserum. However, *A. oryzae* AlPase antiserum reacted more weakly with the heterologous protein (fig. 3).

Fig. 3. Cross-reactions of *A. fumigatus* (F) and *A. oryzae* (O) AlPases with *A. fumigatus* and *A. oryzae* AlPase antisera; 50 ng of protein was transferred on to nitrocellulose membranes and blots were treated with antisera against *A. fumigatus* AlPase (A), *A. oryzae* AlPase (B) and serum from non-immunised rabbits (C). The proteins were detected with alkaline-phosphatase-conjugated goat anti-rabbit IgG.

Discussion

When cultured in soluble collagen as the sole carbon and nitrogen source, *A. fumigatus* secreted a protease to provide nutrients necessary for growth. The enzyme was apparently the major protein secreted by the fungus (fig. 1, track 3). Preparations of the purified *A. fumigatus* and *A. oryzae* AlPases showed two minor bands of 24 and 29, 25 and 30 Kda respectively on SDS-PAGE (fig. 1, tracks 1 and 2). Further gel filtration and ion exchange chromatography (not shown) did not result in preparations showing a unique major band. Since the two minor bands could be eliminated in the preparation from *A. oryzae* by pre-incubation of the enzyme with PMSF, these apparently homologous minor bands are interpreted as being self degradation products of the enzyme. With the same inhibitor, the second band was still present in a preparation of *A. fumigatus* AlPase (fig. 1, tracks 4 and 5.).

Immunoblotting showed that *A. fumigatus* and *A. oryzae* AlPases are closely related, suggesting the existence of common and specific domains in the

enzymes secreted by the two species. However, *A. fumigatus* AIPase differed in having a much higher iso-electric point and a marked peak of activity at pH 9. The *A. fumigatus* AIPase presented a broad spectrum with appreciable activity, and at a physiological pH value of 7.4, the enzyme still had a higher specific activity on collagen and elastin than *A. oryzae* AIPase. The amino acid sequence of *A. oryzae* AIPase shows homology with other serine proteases of the subtilisin family, e.g., *Saccharomyces cerevisiae* protease B, *Yarrowia lipolytica* alkaline extracellular protease, *Tritirachium album* proteinase K and *Bacillus amyloliquefaciens* subtilisin.¹² Enzymes of the subtilisin family are totally inactivated by PMSF whereas TLCK and TPCK, inhibitors of trypsin and chymotrypsin respectively, have no effect.¹³⁻¹⁶ As shown in particular for *S. cerevisiae* proteinase B,¹³ the *A. fumigatus* and *A. oryzae* AIPases were inhibited by PMSF, chymostatin and antipain, but not by iodoacetamide, EDTA, pepstatin, phosphoramidon, E64, SBTI, aprotinin, TLCK and TPCK. Immunoblotting and the effects of various protease inhibitors support the view that the *A. fumigatus* AIPase is a protease of the subtilisin family.

A. oryzae is classified in the "flavus" group of the genus *Aspergillus* which is clearly distinct from the "fumigatus" group.⁴ *A. oryzae* was reported to be the cause of brain lesions in one patient.¹⁷ *A. flavus* is

recognised as causing invasive aspergillosis, but with a frequency which does not approach that of *A. fumigatus*. Like *A. oryzae*, *A. flavus* secretes an AIPase.¹⁸ The high collagenolytic activity and the ability of AIPases to digest elastin can explain the ability of *Aspergillus* spp. to invade and destroy the host tissues. In this sense it has already been shown that all isolates of *A. fumigatus* and *A. flavus* causing aspergillosis produced elastase, but not all isolates producing elastase were associated with invasive disease.¹⁹ Other correlations between protease activity and ability to cause invasive aspergillosis in animals tends to support the view that the pathogenicity of *A. fumigatus* is related to its protease activity.^{2, 3, 20} However, to establish the role of the isolated protease in the virulent behaviour of the fungus, synthesis of this protease must be demonstrated in the infected host and inactivation of the enzyme by gene disruption must be shown to result in reduced pathogenicity.

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