Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue

U. REICHARD, SUSANNE BÜTTNER, H. EIFFERT, F. STAIB* and R. RÜCHEL†

*Department of Medical Microbiology, University of Göttingen and * Division of Mycology, Robert Koch Institute, Bundesgesundheitsamt, Berlin (W), Germany*

Summary. A serine proteinase (Alp) from the culture supernate of a clinical isolate of *Aspergillus fumigatus* was purified to virtual homogeneity at a yield of 41%. The procedure involved affinity chromatography on agarose-ε-amino-caproyl-D-tryptophan methyl ester. Alp had an estimated mol. wt of 32 Kda and the pI was determined at pH 7·9. The enzyme was fully inhibited by phenylmethyl sulphonyl fluoride, chymostatin and α-1-proteinase inhibitor, and it was largely inhibited by α-1-antichymotrypsin. Partial inhibition was observed with tosyl-phenylalanine chloromethyl ketone, but tosyl-lysine chloromethyl ketone was ineffective. Thus, Alp may be identical with the major chymotryptic activity of *A. fumigatus*, which has already been described. The N-terminal sequence of 25 amino acids revealed an 88% homology of Alp with the subtilisin-related proteinase of *A. oryzae*. Alp acted on casein over a broad range from pH 5·5 to 11·5 and also acts to a lesser extent on haemoglobin and serum albumin. The enzyme degraded elastin and a synthetic elastase substrate; hence, it may be identical with the previously described elastinolytic activity of the fungus. At pH 7·3 and a concentration of 1 μg/ml, Alp was not toxic for Vero cells, but it efficiently detached such cells from a plastic surface. Specific antibodies against Alp were detected by enzyme immunoassay in the sera of patients and Alp-antigen was demonstrated by immunofluorescence in mycotic human lung. In addition, a second proteinase (Exalp) with extremely alkaline activity, and an aspartic proteinase of *A. fumigatus* are described.

Introduction

Aspergillus fumigatus is an airborne pathogen; hence, it predominantly afflicts the respiratory system. It can cause allergy (e.g., syndromes related to asthma), colonise pre-existing cavities, leading to aspergilloma and, furthermore, the fungus is a major cause of invasive and disseminated mycosis in severely immunocompromised patients (see reviews [1-3]).

In spite of its medical importance, *A. fumigatus* accounts for only a small proportion of the airborne mould spores.[2] Therefore, it is likely that this fungal species possesses specific virulence factors which distinguish it from other non-pathogenic moulds.

Aspergilli are rich in extracellular proteases[4,5] and it has been suggested repeatedly that such enzymes are involved in the pathogenesis of aspergillosis.[6-9] The invasion of blood vessels by *A. fumigatus*[2] suggest that there is fungal proteolytic activity during infection, and the isolate used in our investigation was derived from a case of lethal haemoptysis.[10]

A. fumigatus produces various extracellular proteolytic enzymes,[4,5] but our attention was focused on a chymotrypsin-related enzyme which must be produced during infection, because it gives rise to a specific immune response in patients.[11] The characterisation of this enzyme may shed some light on its role in the pathogenesis of aspergillosis and on its potential as a diagnostic marker.

Materials and methods

Organism

A. fumigatus strain D141 was derived from a 45-year-old male patient with a history of pulmonary tuberculosis

who died from massive haemoptysis complicating aspergilloma. Before death, a typical (green) isolate of *A. fumigatus* (strain D141), and an atypical (white) isolate were obtained from respiratory secretions.[10] Strain D141 was strongly proteolytic and it hydrolysed the proteins of pleural fluid *in vitro*.[12]

Growth media

Glucose Peptone Broth (Merck, Darmstadt, Germany) was prepared as a 2% liquid medium. YCB-casein medium contained casein (Merck) 10 g/L, glucose 10 g/L and Yeast Carbon Base (Difco; YCB) 11·7 g/L; it was adjusted to pH 7·6 and sterilised by filtration. YCB-haemoglobin medium was prepared with bovine haemoglobin (Sigma) instead of casein.

General proteolytic activity

General proteolytic activity of culture supernate was determined by the casein assay[13] or with bovine haemoglobin or bovine serum albumin (Sigma, no. A790; BSA), 5 g/L as substrate. For the azocasein assay,[14] azocasein (Sigma) 5 g/L was dissolved in 0·1 M Tris-HCl buffer at pH 8; 400 μl of this substrate solution were mixed with 10–100 μl of the enzyme sample. After incubation at 37°C, 150 μl of trichloroacetic acid 20% were added. After 30 min, the precipitate was centrifuged at 8000g for 2 min. The supernate was mixed with the same volume of 1 N NaOH, and absorption of liberated dye was measured at 436 nm. The activity was expressed in arbitrary units. One azocasein unit was defined as an increase of 0·1 absorption unit after incubation for 1 h at 37°C. Acid proteolytic activity was determined at pH 3 in 0·1 M citrate buffer with haemoglobin as a substrate.

Elastinolytic activity

Elastin congo red (Sigma; 1 g/L in 0·2 M sodium borate buffer, pH 8) was used as substrate for monitoring elastinolytic activity. The activities of Alp and porcine elastase (Sigma) were compared at pH 8 by monitoring the time required to liberate 50% of the bound dye from the substrate.

Synthetic peptide substrates

The hydrolysis of N-acetyl-L-arginine (Serva, Heidelberg, Germany), tyrosine (Serva) and alanine (Sigma)-*p*-nitroanilides was monitored essentially according to the method of Kraus and Femfert[15] in 0·1 M Hepes buffer (Biochrom, Berlin, Germany), pH 7, with 1 mM $CaCl_2$ and dimethylformamide 5% v/v. The final concentration of Alp in the reaction mixture was in the range 10^{-6} M. The absorption of liberated *p*-nitroaniline was determined photometrically at 405 nm. The hydrolysis of succinyl-alanine-alanine-proline-phenylalanine-*p*-nitroanilide (Sigma) was monitored similarly, with a stock solution of

10^{-2} M in dimethylsulphoxide. The hydrolysis of N-CBZ-L-alanine-*p*-nitrophenyl ester (Sigma) (from a 4×10^{-2} M stock solution in acetonitrile) was monitored in the same buffer as above. The final concentration of Alp (or porcine elastase) was 5×10^{-9} M. The absorption of liberated *p*-nitrophenol was determined photometrically at 400 nm. The Michaelis constant K_M was determined from the Lineweaver-Burk plot. At least four different substrate concentrations between 10^{-2} and 2×10^{-5} M were used.

Inhibition of proteinase activity

Phenylmethylsulphonyl fluoride (Serva; PMSF) was used from a stock solution of 0·1 M in ethanol. At a final concentration of 10^{-4} M it was treated with proteinase for 20 min at room temperature in 50 mM phosphate buffer, pH 7·4. Chymostatin (Peptide Institute, Osaka, Japan) was made up at 1 g/L in dimethylsulphoxide. This stock solution was used at a 100-fold dilution in the proteinase assay.

Pepstatin (Peptide Institute) was made up in methanol at a concentration of 10^{-3} M. This stock solution was used at a 100-fold dilution in the proteinase assay. Tosyl lysine chloromethyl ketone (Sigma; TLCK) was dissolved at 5 g/L in water immediately before use. This stock solution was diluted 10-fold in the proteinase solution (in 50 mM phosphate buffer, pH 7·4) and was left for 1 h at room temperature before assay of residual enzymic activity. Tosyl phenylalanine chloromethyl ketone (Sigma; TPCK) at a concentration of 28 mM was dissolved in methanol. The stock solution was used as described for TLCK.

Iodoacetamide (Serva), a blocking agent of sulphydryls, at a concentration of 10^{-3} M, was allowed to react with proteinase in 0·1 M Tris-HCl buffer, pH 8. Residual proteolytic activity was determined after 20 min at room temperature with haemoglobin as substrate. Mercuric chloride (Sigma) was used at a concentration of 10 mM in 0·1 M Tris-maleate buffer, pH 7 with N-CBZ-L-alanine-*p*-nitrophenyl ester (Sigma) as substrate. Human α-1-proteinase inhibitor (Sigma) and α-1-anti-chymotrypsin (Calbiochem, San Diego, CA, USA) (both 10^{-6} M) were mixed with proteinase in 50 mM Tris-HCl buffer, pH 8. After 3 min at room temperature, residual enzymic activity was determined by casein assay.

Chromatography

DEAE-chromatography with DEAE-Sephadex-A50 (Pharmacia AB, Uppsala, Sweden) and gel filtration on Sephadex G25 were performed according to standard protocols; carboxymethyl Sephadex (CM-Sephadex-C50) was used according to the methods of Martin and Jönsson.[16] Chromatography on ε-aminocaproyl-D-tryptophan-methylester agarose (ICN-Immuno Biologicals, Lisle, Il, USA) was performed according to the method of Bout *et al.*[17]; the column (10 × 1 cm) and the sample were adjusted to 0·05 M Tris-HCl, pH 8·7. The sample was adsorbed to the gel for 2 h at 6°C. Subsequently, the

column was rinsed with three volumes of the same buffer and the proteinase was desorbed with acetic acid (0·1 M). Immediately after desorption, fractions with proteinase were titrated back to pH 5 with 2 M sodium acetate buffer. The protein concentration was monitored photometrically at 280 nm.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in continuous gel gradients from 2·5 to 20% total acrylamide at a constant cross linkage of 5% was performed as previously described.[18] The gradient gels were also used for sodium dodecyl sulphate (SDS)-PAGE: the samples were made up with SDS and dithiothreitol (Serva) at the indicated concentrations and were usually boiled for 2 min; the buffer at the cathode was made up with SDS to 0·1%.

Iso-electric focusing in granulated dextran gel (IEF-Sephadex, Pharmacia) was performed, with the water cooled Desaphor equipment and carrier ampholytes, pH 2–11, 2% v/v from Serva.

Sequencing of amino acids

Partial amino acid sequencing was accomplished by in-situ Edman degradation[19] in the laboratory of Dr H. D. Kratzin, Max-Planck Institute für Experimentelle Medizin, Göttingen.

Immunisation schedule

Two non-inbred adult female guinea-pigs were given four doses of 30 μg of purified Alp (in 30 mM acetate buffer at pH 4·8) by subcutaneous injection at intervals of 2 weeks. For the first injection, Alp was made up in an equal volume of complete Freund's adjuvant; incomplete adjuvant was used for subsequent immunisation. The animals were bled 10 days after the last injection. γ-Globulins were separated from the sera by sodium sulphate precipitation. Specific antibodies were monitored by enzyme-linked immunosorbent assay.

Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA protocol was used for monitoring anti-Alp antibodies in human and guinea-pig sera.[20] Polystyrene micro-test plates were coated overnight at 4°C with purified Alp 5 μg/ml in phosphate-buffered saline (PBS, pH 7·4) containing the proteinase inhibitor PMSF at a concentration of 10^{-4} M; subsequently, the rinsed plates were saturated with PBS containing BSA 10 g/L and Tween-20 0·1%. After addition of the human or guinea-pig serum and rinsing with PBS, anti-Alp antibodies were detected with anti-human and anti-guinea-pig immunoglobulin-peroxidase conjugates (Dako). The serum samples and the commercial second antibody-peroxidase conjugates were diluted 1000-fold before use.

Immunofluorescence

Alp-antigen was demonstrated in formaldehyde-fixed tissue after deparaffinisation. Non-specific reactivity was suppressed by 2000-fold dilution of anti-Alp serum from guinea-pig in PBS containing BSA 10 g/L. Anti-guinea-pig immunoglobulin-fluorescein conjugate (Dako) was diluted 1000-fold in the same medium. Before fluorescence microscopy, the tissue section was embedded in PBS-glycerol containing *p*-phenylenediamine (Sigma) to reduce fluorescence fading.[21]

Cell culture

Vero (monkey kidney) cells were kindly provided by Ms K. Engelhardt of this institution. They were cultured in Instamed RPMI 1640-199 Earle tissue culture medium (Biochrom) supplemented with fetal calf serum (Biochrom) 1%,[22] using flat-bottomed polystyrene flasks and an incubation atmosphere of CO_2 5% in air. The viability of cells was judged by the uptake of neutral red, or trypan blue (Sigma).

Results

Monitoring and purification of Alp

The profile of extracellular proteolytic activity of *A. fumigatus* strain D141 was monitored over 10 days during growth at 37°C in haemoglobin medium under aerobic conditions (fig. 1). Acid proteolytic activity (pH 3) against haemoglobin reached a maximum between the fifth and eighth day and thus reflected the profile of the pH of the medium. The alkaline proteolytic activity (pH 8), which was determined with azocasein as substrate, reached a maximum around the third day of culture.

According to this profile, for purification of alkaline protease, medium from a casein culture was harvested when the pH had dropped to a value of 5. In a typical experiment at this stage of the culture, the medium contained an activity of 13·4 (arbitrary) azocasein units/ml. As was deduced from the activity of purified enzyme, this corresponded to a concentration of 2·2 μg/ml.

The medium was saturated to 80% with ammonium sulphate, which caused the precipitation of the proteolytic activities. The precipitate was dissolved and dialysed against distilled water and was adjusted to a conductivity of 300 μSi/cm by further addition of water. Subsequently a major fraction of alkaline proteolytic activity (Alp) was adsorbed by a batch procedure to carboxymethyl Sephadex at pH 6·8 in 2 mM sodium potassium phosphate buffer. The acid proteolytic activity (and a second alkaline proteinase, Exalp) were separated from Alp at this stage (see below). The desorption
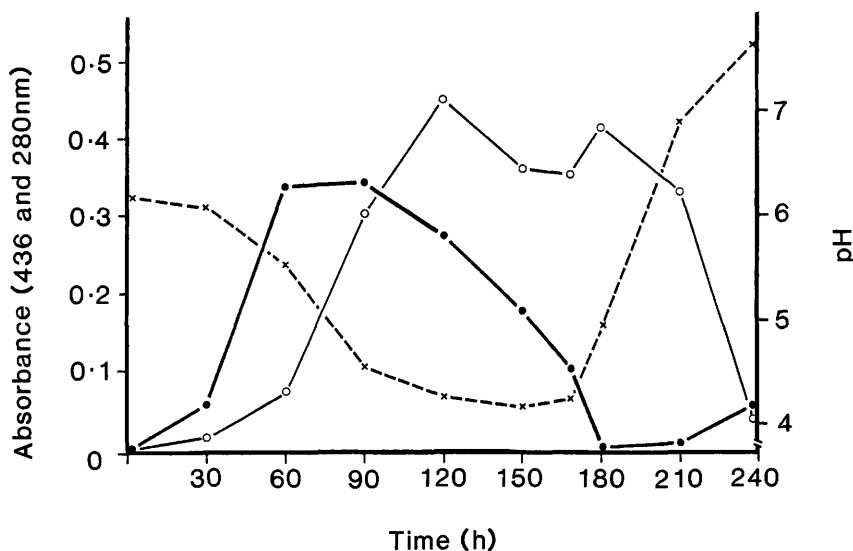
Fig. 1. Profile of proteolytic activity in the culture medium of *A. fumigatus*. Alkaline activity (—●—; A_{436}) was monitored by the azocasein assay at pH 8 and acidic activity (—○—; A_{280}) by the haemoglobin assay at pH 3. The activities were measured photometrically. The pH of the medium (×---×) was monitored over 240 h.

of Alp from the cation exchanger was accomplished with the starting buffer after addition of 0·2 M sodium chloride. The pooled fractions with Alp activity were subjected twice to nitrogen pressure dialysis (Amicon membrane PM-10), which raised the specific activity by a factor of 43. This effect may reflect the filtration of large quantities of residual casein fragments. Finally, Alp was subjected to affinity chromatography with ε-amino-caproyl-D-tryptophan-methylester agarose at pH 8·7 as outlined above. The eluate was immediately titrated to pH 4·8 with 2 M sodium acetate buffer and was transferred to 30 mM sodium acetate buffer, pH 5·0, by gel filtration through Sephadex G-25. The fractions of the finale eluate contained 41% of the starting activity at a specific activity which was raised 2558-fold above the initial level. The Alp preparation was divided into small volumes which were stored at −18°C without appreciable loss of activity.

Properties of Alp

When purified Alp was subjected to SDS-PAGE, a polymorphism of protein peaks was observed (fig. 2a). The inconsistency of this pattern suggested the involvement of autolytic processes, which was proved by the effect of pre-treating the sample with the proteinase inhibitor PMSF (fig. 2b). Parallel electrophoresis of standard proteins suggested a mol. wt for Alp around 32×10^3 (fig. 2c). The

relative position of the Alp peak was affected neither by pre-treatment with reducing agent (20 mM dithiothreitol), nor by subsequent treatment with 20 mM iodoacetamide. The iso-electric point of the enzyme was determined to be pH 7·9 by electro-focusing in granulated dextran gel, subsequent elution of gel fractions, and measurement of pH and proteolytic activity of the eluates.

The N-terminal fraction of Alp was directly sequenced by in-situ Edman degradation of the protein after SDS-PAGE and Western blotting on polyvinylidene-difluoride (PVDF) membrane (Millipore). The elucidated sequence of 25 amino acids was largely (88%) homologous with the corresponding sequence of the alkaline proteinase of *A. oryzae*. An exchange of amino acids was observed only in position 1 (alanine for glycine), position 7 (glycine for serine), and position 21 (alanine instead of glutamine).

Substrates of Alp

Alp degrades casein between pH 5·5 and 11·5; the maximum activity was observed at pH 9·5 (fig. 3). The acid tolerance of Alp varied with the temperature. Thus, denaturation was observed at pH 3, 6°C (0·2 M glycine-HCl buffer), at pH 4·2, 20°C and pH 4·5, 37°C (0·2 M citrate-HCl buffer, 60 min).

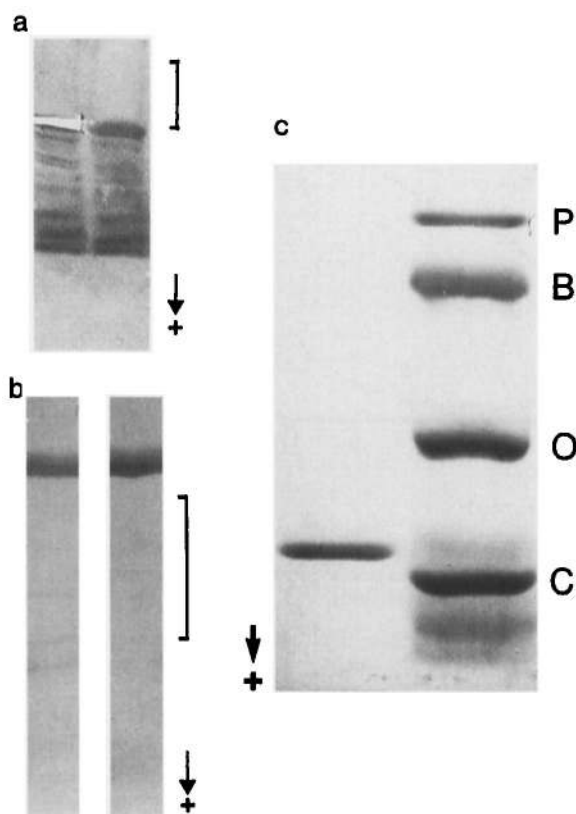At pH 8, Alp degraded bovine haemoglobin and bovine serum albumin (26% and 17%, respectively,

tyl-L-tyrosine at K_m-values of $6\cdot3\times10^{-3}$, $1\cdot5\times10^{-2}$, and $6\cdot7\times10^{-4}$ M, respectively.

Resistance to SDS and reducing agent

Alp resisted exposure to SDS 0·06% 37°C for 60 min; at SDS 0·12%, it lost 40% of its activity, and at SDS 0·5% total inactivation was observed. Residual enzymic activity was monitored at pH 7 with Succ-Ala-Ala-Pro-Phe-pNa as a substrate. Under similar conditions, Alp tolerated at least 0·1 M dithiothreitol.

Inhibition of Alp

Purified Alp was exposed to various inhibitors (table). The enzyme was fully inhibited by PMSF, chymostatin, and α-1-proteinase inhibitor and was largely inhibited by α-1-anti-chymotrypsin. Therefore, Alp is a serine proteinase with functional relationship to chymotrypsin. This relationship was supported by partial inhibition with TPCK and by lack of sensitivity to TLCK. The conflicting results of exposure to iodoacetamide (no inhibition), and mercuric chloride (92% inhibition) do not allow any conclusion concerning the presence of sulphydryls in Alp.

Fig. 2(a) Autolytic degradation of purified proteinase (Alp): Coomassie Blue pattern of autolytic fragments after pretreatment of Alp with SDS 3% + 320 mM DTT (100°C, 3 min), SDS-PAGE, and subsequent Western blotting on a PVDF-membrane. The protein band with highest molecular weight was excised for sequencing. Bar = 1 cm. **(b)** 1: Coomassie Blue pattern of Alp and autolytic fragments after treatment with SDS 3% (100°C, 1 min), and SDS-PAGE. 2: Pattern of Alp after pretreatment with 0·1 mM PMSF (20°C, 20 min) and SDS-PAGE as above. Bar = 1 cm. **(c)** Coomassie Blue pattern of PMSF-treated Alp (1), and standard proteins (2) after SDS-PAGE. The estimated mol. wt of Alp is 32×10^3. P: phosphorylase-b (97·4); B: bovine serum albumin (66·2); O: ovalbumin (42·7); C: carboanhydrase (31).

Effect of purified Alp on cultured mammalian cells

Log phase cultures of adherent Vero cells at pH 7·3 were exposed to purified Alp at concentrations of 1 or 5 µg/ml in culture medium containing fetal calf serum 1% v/v. Detachment of a majority of the cells was obvious after 10 and 4 h, respectively. As judged by the uptake of the vital stain neutral red, the detached cells remained viable (fig. 4).

Under similar conditions, Alp was directly compared with bovine trypsin (Sigma). At an identical concentration of 3 µg/ml, half of the Vero

of the caseinolytic activity under similar conditions). Purified Alp is weakly elastinolytic. Under comparable conditions at pH 8 (as outlined above), Alp caused 2% of the hydrolysis of elastin congo red compared with the effect of porcine elastase. The chromogenic elastase substrate N-CBZ-L-alanine-p-nitrophenyl ester was efficiently hydrolysed by Alp at pH 7 (Km $4\cdot2\times10^{-4}$ M); the corresponding value for porcine elastase was $1\cdot5\times10^{-4}$ M.

Alp also hydrolysed the 4-nitroanilides of N-acetyl-L-arginine, N-acetyl-L-alanine, and N-ace-

Table. Inhibition of Alp

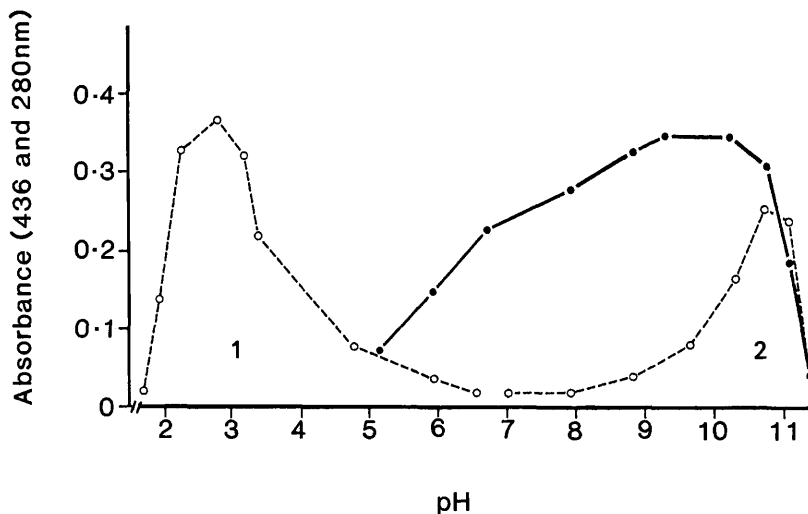| Inhibitor	Concentration (M)	pH	Inhibition (%)
PMSF	10^{-4}	7·4	100
Chymostatin	$1\cdot7\times10^{-5}$	8	100
TPCK	$2\cdot8\times10^{-3}$	7·4	30
TLCK	$1\cdot4\times10^{-3}$	7·4	0
Iodoacetamide	10^{-3}	8	0
HgCl$_2$	10^{-2}	7	92
α-1-proteinase inhibitor	10^{-6}	8	94
α-1-anti-chymotrypsin	10^{-6}	8	73

Fig. 3. Proteolytic activity after chromatography on CM-Sephadex. The activity of Alp in the eluate (—●—) was monitored at the indicated pH-values with casein as substrate. The non-adsorbed activity (—○—) of acidic proteinase (1) was monitored with haemoglobin as substrate, and non-adsorbed Exalp (2) was monitored with casein as substrate.

cells were detached by Alp after 3 h, whereas trypsin did not cause recognisable detachment during observation for 20 h. As judged by the criterion of trypan blue uptake, the detached cells were viable, and consequently, they could be propagated in culture.

Antibodies to Alp

Purified Alp was used for immunisation of two guinea-pigs as outlined above. Ten days after the third challenge, the animals were bled, and the titres of specific antibodies were determined by ELISA at 50 000 and 100 000, respectively. The
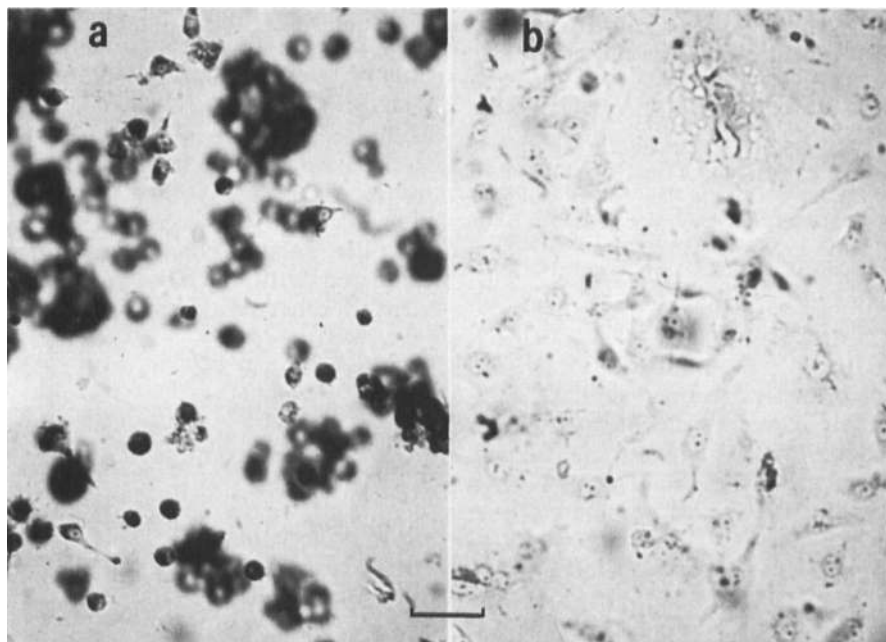

Fig. 4. Detachment by Alp of cultured Vero cells from a polystyrene surface. (a) Log-phase cells after exposure to Alp 5 μg/ml for 4 h in culture medium. The detached round cells took up neutral red, which indicates their viability. (b) Control cells without proteinase; the cells are still adhering to the polystyrene. Bar = 30 μm.

presence of the proteinase inhibitor PMSF (10^{-4} M) was required during coating of the micro-test plate with Alp. Without PMSF, the results of ELISA were irreproducibly diminished by 20–50%.

By the same indirect ELISA, specific antibodies were also detected in the sera of two out of six patients with proven pulmonary colonisation by *Aspergillus*. The titres were 15 000 and 30 000, respectively.

Demonstration of Alp-antigen in situ

The guinea-pig immune serum was applied to sections of formaldehyde-fixed lung from a patient who had died of invasive aspergillosis. At a 2000-fold dilution of the serum, fungal elements, including hyphae, conidiophors and conidia, showed a positive reaction for the Alp-antigen (fig. 5), whereas parallel exposure of an adjacent tissue section to pre-immunisation serum under identical conditions did not produce a positive reaction.

Other proteinases of A. fumigatus

Another alkaline proteinase (Exalp), and an acid proteinase were identified in the medium of strain D141 after culture for 3 days at 37°C, when the pH had dropped to 4·8. Both enzymes were detected in the void volume of cation exchange chromatogra-

phy at pH 6·8. Exalp and the acid proteinase could be separated by chromatography on DEAE-Sephadex, which was equilibrated with 10 mM phosphate buffer, pH 6·8. Exalp desorbed from the column after addition of 0·2 M sodium chloride and the acid proteinase followed at 0·5 M sodium chloride.

The acid proteinase was fully sensitive to pepstatin at pH 3; thus, it was a typical aspartic proteinase. Exalp was fully inhibited by chymostatin at pH 10·8; it was not affected by PMSF under these conditions. The optimum of caseinolytic activity of Exalp was determined at pH 10·8. Electrofocusing revealed a pI of Exalp in the pH range 5–6.

Discussion

The extracellular proteolytic activity of aspergilli was reviewed as early as 1950,[23] and Jönsson and Martin[6] first considered a possible role of the enzymes in the pathogenesis of aspergillosis. The authors suggested the existence of at least two proteolytic exoenzymes from *A. fumigatus* with acidic and alkaline optima of activity. The purification of the alkaline activity was attempted by cation exchange chromatography. However, the homogeneity of the resulting fractions was not proven.[16] Later, evidence for at least two chymotrypsin-related exoenzymes of *A. fumigatus* was
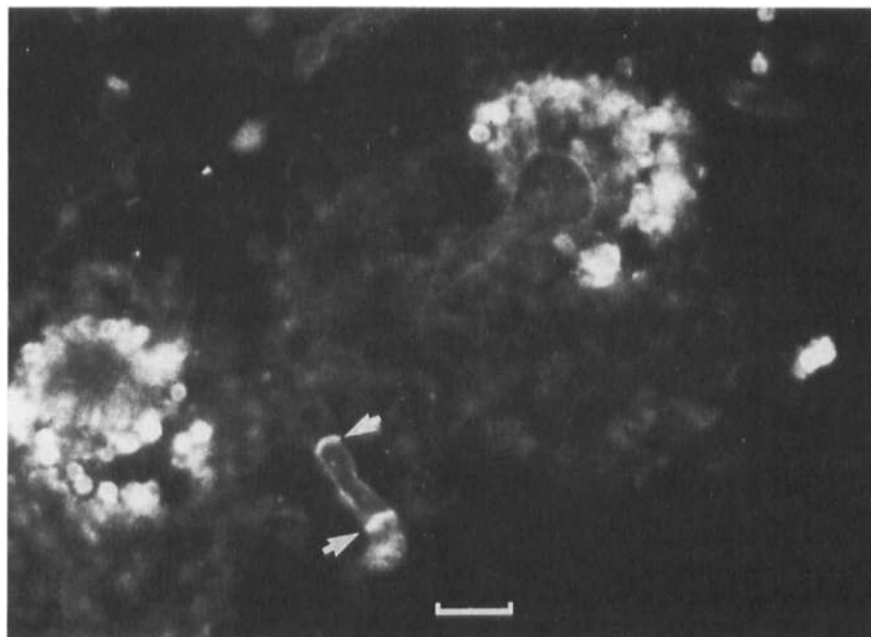

Fig. 5. Aspergilli in human lung from a fatal case of aspergillosis. The fungal elements were stained by immunofluorescence for fungal protease (Alp) with a fluorecein label. Note the strong antigenicity of the conidia and the septate hyphae (arrow). Bar = 20 μm.

presented on the basis of immuno-electrophoresis.[24] The heterogeneity of one of these enzymes may be interpreted as a sign of autolysis, which leads to enzymically active fragments (unpublished data).

The purification of a chymotrypsin-related enzyme was attempted subsequently by Bout et al.[17] who used D-tryptophan methylester as an immobilised ligand in affinity chromatography. The immunological analysis of their product revealed five distinct molecular species (which may again reflect the autolytic activity of the enzyme). In 1984, Schønheyder and Andersen[25] reported a neutral proteolytic activity in the culture filtrate of A. fumigatus which was partially purified by hydrophobic interaction chromatography. Schønheyder[1] also suggested that the caseinolytic activity of these fractions represented three individual enzymes. In addition, Kothary et al.[9] reported an inducible elastinolytic activity of strains of A. fumigatus which were virulent in mice. The results were confirmed by Rhodes et al.,[26] who found elastinolytic activity in all aspergilli from clinical cases of invasive mycosis.

In spite of the different approaches, purification to homogeneity of the neutral or alkaline proteinases of A. fumigatus has not been accomplished. Only the extracellular acid proteinase has been characterised in some detail.[27] This enzyme was fully inhibited by pepstatin; therefore, it is an aspartic proteinase (E.C.3.4.23).

In addition, we have identified a proteinase (Exalp) with an extremely alkaline optimum of caseinolytic activity. The classification of this enzyme remained uncertain. It was inhibited by chymostatin and insensitive to PMSF. Exalp also resisted di-isopropyl fluorophosphate (R. Zech, personal communication).

However, the proteolytic activity attracting our attention was the caseinolytic activity (Alp), which is demonstrable over a broad range from weakly acidic to alkaline pH. This property may permit enzymic activity in the infected host. We have purified Alp and demonstrated its homogeneity after pre-treatment with PMSF. This indicates the autolytic potential of the enzyme and explains, at least in part, the conflicting data presented in previous reports which have alluded to up to five chymotrypsin-related enzymes of A. fumigatus.[17] The N-terminal amino acid sequence revealed a homology of 88% with the sequence of the alkaline proteinase "Alp" of A. oryzae, a typical serine proteinase of the subtilisin-type.[28] We also observed 39% homology with proteinase-K from Tritirachium album.[29] The relationship to proteinase-K is also reflected by the relative resistance of Alp to sodium dodecyl sulphate; however, Alp hydrolysed the 4-nitroanilide of arginine, whereas proteinase-K did not.[15] There was no evidence for disulphide bonds in Alp, which is in agreement with the related enzyme of A. oryzae,[28] whereas proteinase-K has two cystins.[29]

Alp was found to possess slight elastinolytic activity, and it may be related to the elastase of A. fumigatus, which was described previously.[9,26] The elastinolytic and caseinolytic activity of this enzyme proved to be inseparable, and the final purification product, examined by SDS-PAGE under reducing conditions, consisted of three molecular species of mol. wt $<35 \times 10^3$.[30] These findings, and the outlined inhibition pattern, largely agree with the properties of Alp.

Immune responses to chymotrypsin-related antigens of A. fumigatus have been observed previously.[11,25,31] These antigens were labelled CHII, C-antigen, or Ag13, respectively; essentially, they may consist of Alp. Specific antibodies to Alp were demonstrable in sera of patients with known pulmonary colonisation by A. fumigatus, and Alp-antigen was detectable in mycotic human lung. Thus, Alp could be an antigenic marker for the acute stage of invasive aspergillosis.

REFERENCES

1. Schønheyder H. Pathogenetic and serological aspects of pulmonary aspergillosis. Scand J Infect Dis 1987; Suppl 51: 1–62.

2. Bodey GP, Vartivarian S. Aspergillosis. Eur J Clin Microbiol Infect Dis 1989; 8: 413–437.

3. Walsh TJ, Dixon, DM. Nosocomial aspergillosis: environmental microbiology, hospital epidemiology, diagnosis and treatment. Eur J Epidemiol 1989; 5: 131–142.

4. Nakagawa Y. Alkaline proteinases from Aspergillus. Methods Enzymol 1970; 19: 581–591.

5. Cohen BL. The proteases of aspergilli. In: Smith JE, Pateman JA (eds) Genetics and physiology of Aspergillus. London, Academic Press. 1977: 281–292.

6. Jönsson AG, Martin SM. Protease production by Aspergillus fumigatus. Agr. Biol Chem 1964; 28: 734–739.

7. Miyaji M, Nishimura K. Relationship between proteolytic activity of Aspergillus fumigatus and the fungus invasiveness of mouse brain. Mycopathologia 1977; 62: 161–166.

8. Staib F. Extracellular proteolytic activity of *Aspergillus fumigatus* strains with septum-like structures in their phialides in serum-albumin agar. *Zentralbl Bakteriol Mikrobiol Hyg* [A] 1982; **252**: 279–285.

9. Kothary MH, Chase T, 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**: 320–325.

10. Staib, F, Mishra SK, Rajendran C *et al*. A notable aspergillus from a mortal aspergilloma of the lung. New aspects of the epidemiology, serodiagnosis and taxonomy of *Aspergillus fumigatus*. *Zentralbl Bakteriol* [A] 1980; **247**: 530–536.

11. Biguet J, Tran van Ky P, Fruit J, Andrieu S. Identification d'une activité chymotrypsique au niveau de fractions remarquables de l'extrait antigénique d'*Aspergillus fumigatus*. Repercussions sur le diagnostic immunologique de l'aspergillose. *Rev Immunol (Paris)* 1967; **31**: 317–328.

12. Staib F. Pleural fluid as nutrient substratum for *Aspergillus fumigatus* and *Aspergillus flavus*. Submerged growth in pleural fluid and extracellular proteolysis in pleural fluid agar. *Zentralbl Bakteriol Mikrobiol Hyg* [A] 1985; **260**: 543–549.

13. Kunitz M. Crystalline soybean trypsin inhibitor. *J Gen Physiol* 1947; **30**: 291–310.

14. Scharmann W, Balke E. Untersuchungen über eine Protease (Elastase) von *Pseudomonas aeruginosa*. I. Bildung und Reinigung der Protease. *Hoppe Seylers Z Physiol Chem* 1974; **355**: 443–450.

15. Kraus E, Femfert U. Proteinase K from the mold *Tritirachium album limber*. Specificity and mode of action. *Hoppe Seylers Z Physiol Chem* 1976; **357**: 937–947.

16. Martin SM, Jönsson AG. An extracellular protease from *Aspergillus fumigatus*. *Canad J Biochem* 1965; **43**: 1745–1753.

17. Bout D, Fruit J, Capron A. Application de la chromatographie d'affinité à l'isolement des fractions antigéniques d'*Aspergillus fumigatus* supportant une activité chymotrypsique. *C R Acad Sci [D] (Paris)* 1973; **276**: 2341–2344.

18. Rüchel R, Gross J. Preparations of continuous gradient gel slabs: a simple technique. *Anal Biochem* 1979; **92**: 91–98.

19. Kratzin HD, Wiltfang J, Karas M, Neuhoff V, Hilschmann N. Gas-phase sequencing after electroblotting on polyvinylidene difluoride membranes assigns correct molecular weight to myoglobin molecular weight markers. *Anal Biochem* 1989; **183**: 1–8.

20. Haun U, Rüchel R, Spies A. A series of serological tests for the detection of antigens and specific antibodies in deep-seated candidosis: experimental aspects. *Mykosen.* 1987; **30**: 472–482.

21. Johnson GD, Davidson RS, McNamee KC, Russel G, Goodwin D, Holborow EJ. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *J Immunol Methods* 1982; **55**: 231–242.

22. Sundsmo JS, Götze O. Human monocyte spreading induced by factor B of the alternative pathway of complement activation. *Cell Immunol* 1980; **52**: 1–17.

23. Dion WM. The proteolyic enzymes of microorganisms. I. Survey of fungi and actinomycetes for protease production in submerged culture. *Canad J Res [C]* 1950; **28**: 577–585.

24. Tran van Ky P, Uriel J, Rose F. Charactérisation de types d'activités enzymatiques dans des extraits antigeniques d'*Aspergillus fumigatus* après électrophorèse et immunélectrophorèse en agarose. *Ann Inst Pasteur (Paris)* 1966; **111**: 161–170.

25. Schønheyder H, Andersen P. Fractionation of *Aspergillus fumigatus* antigens by hydrophobic interaction chromatography and gel filtration. *Int Archs Allergy Appl Immunol* 1984; **73**: 231–236.

26. Rhodes JC, Bode RB, McCuan-Kirsch CM. Elastase production in clinical isolates of *Aspergillus*. *Diagn Microbiol Infect Dis* 1988; **10**: 165–170.

27. Panneerselvam M, Dhar SC. Physico-chemical properties of the acid proteinase from *Aspergillus fumigatus*. *Ital J Biochem* 1981; **30**: 63–74.

28. Tatsumi H, Ogawa Y, Murakami S *et al*. A full length cDNA clone for the alkaline protease from *Aspergillus oryzae*: structural analysis and expression in *Saccharomyces cerevisiae*. *Mol Gen Genet* 1989; **219**: 33–38.

29. Jany KD, Lederer G, Mayer B. Proteinase K—a new subclass of the subtilisins. The amino acid sequence of the enzyme. *Biol Chem Hoppe Seyler* 1986; **367**: 87.

30. Frosco M, Chase T, Macmillan J. Further purification of elastase from *Aspergillus fumigatus*. Annual Meeting of the American Society for Microbiology 1989, New Orleans. Abstract No. F 88.

31. Harvey C, Longbottom JL. Characterization of a second major antigen Ag_{13} (antigen C) of *Aspergillus fumigatus* and investigation of its immunological reactivity. *Clin Exp Immunol* 1987; **70**: 247–254.