

Molecular characterization and influence on fungal development of ALP2, a novel serine proteinase from *Aspergillus fumigatus*

Utz Reichard^{1,2}, Garry T. Cole¹, Terry W. Hill^{1,3}, Reinhard Röchel² and Michel Monod⁴

¹ Department of Microbiology and Immunology, Medical College of Ohio, Toledo, OH 43614, USA

² Department of Medical Microbiology, University of Göttingen, Göttingen, Germany

³ Department of Biology, Rhodes College, Memphis, TN 38112, USA

⁴ Laboratoire de Mycologie, Service de Dermatologie, Centre Hospitalier Universitaire Vaudois, 1011 Lausanne, Switzerland

Received May 19, 2000 · Revision received July 13, 2000 · Accepted August 3, 2000

Abstract

A novel subtilisin-related serine proteinase (ALP2) [EC 3.4.21.48] with a broad range of activity between pH 4.5 and 11.0 was released from a cell wall fraction of *Aspergillus fumigatus* by an alkaline pH shift. The enzyme which was not detected in the culture supernatant was partially purified by phenylbutylamine agarose chromatography. The N-terminal sequence revealed that ALP2 is the same protein identified as the major allergen of *A. fumigatus* in patients suffering from extrinsic bronchial asthma (Shen et al. 1999, Int. Arch. Allergy Immunol. 119, 259–264). Based on this N-terminal sequence and on a conserved region of fungal subtilisins, a specific PCR probe was generated and the ALP2 genomic and cDNA were isolated from corresponding phage libraries. ALP2 shares a 49 % identity with the vacuolar proteinase B (PrB) of *Saccharomyces cerevisiae*. In addition there is a 78 % identity with PEPC, a serine proteinase which has been described in *Aspergillus niger*. Targeted disruption of the ALP2-encoding gene resulted in a slightly decreased speed of vegetative growth and in a more than 80 % reduction of sporulation in the *alp2*-negative mutants, correlated with an approximately 50 % reduction of the median diameter of conidiophore vesicles. The requirement of ALP2 for regular sporulation, in addition to its role in allergic asthma, raises further interest in cellular proteinases in respect to morphogenesis and pathogenesis in *A. fumigatus*.

Key words: proteinase – serine proteinase – vacuolar proteinase – vacuole – cell wall – ALP2 – fungus – *Aspergillus fumigatus*

Introduction

Filamentous fungi of the genus *Aspergillus* are known to cause invasive aspergillosis (IA) in the immunocompromised host (Denning, 1998; Latgé, 1999; Röchel and Reichard, 1999), aspergillomata in immunocompetent patients mostly with underlying lung diseases

like tuberculosis (Röchel and Reichard, 1999), and allergic diseases in patients suffering from asthma or cystic fibrosis (Cockrill and Hales, 1999; Crameri, 1999). Because of their potential role in pathogenesis in respect to invasiveness of fungal hyphae into the host tissue, so far three extracellular endoproteinases of *A. fumigatus* (ALP1, MEP, and PEP1) have been in-

Corresponding author: Dr. Michel Monod, Laboratoire de Mycologie, Service de Dermatologie, CHUV, CH-1011 Lausanne, Switzerland, Phone: +41 21 314 0376, Fax: +41 21 314 0378, E-mail: Michel.Monod@chuv.hospvd.ch

vestigated in detail (Lee and Kolattukudy, 1995; Monod et al., 1991, 1993; Reichard et al., 1990, 1994). Animal trials with knock-out mutants did not support a significant contribution of the proteinases to the pathogenesis of invasive aspergillosis (Jaton-Ogay et al., 1992, 1994; Reichard et al., 1997; Tang et al., 1993). However, these proteinases secreted by *A. fumigatus* seem to be involved in the pathogenesis of allergic disorders due to the fungus (Chow et al., 2000; Cramer, 1998; Cramer and Blaser, 1996; Shen et al., 1999).

A new interest in the proteinases of *A. fumigatus* was raised with the finding of proteinases located in the cell wall fraction. These proteinases active in the acid and alkaline pH range are different from the endoproteinases secreted by the fungus. Recently, we have purified and characterized an aspartic proteinase (PEP2) with homology to the vacuolar proteinase PrA of *Saccharomyces cerevisiae* (Reichard et al., 2000). Until now, few reports on cell wall-associated proteinases of medically important fungi have been published and their function still remains unclear. In the present work, we report the isolation and characterization of a novel serine proteinase, ALP2, which contributes to the proteolytic activity of the *A. fumigatus* cell wall fraction in the alkaline pH range. Like PEP2, ALP2 exhibits homology to a yeast vacuolar proteinase, in this case PrB. In addition, ALP2 turned out to be identical with the recently identified major allergen of *A. fumigatus* associated with extrinsic bronchial asthma (Shen et al., 1999), and we present data that ALP2 contributes significantly to efficient conidia production in this fungus.

Materials and methods

Strains and culture conditions

For extraction of native proteinase, a clinical isolate of *Aspergillus fumigatus* strain D141 (Reichard et al., 1997) was grown on Sabouraud glucose agar plates for 3 days at 37°C. Conidia in 10 ml of saline containing 0.1 % Tween 80 (Sigma, St. Louis, MO) were used for inoculation of 2-l Erlen-

meyer flasks containing 350 ml of minimal medium (Cove, 1966). Aerated flasks were incubated for 16 to 24 hours at 37°C with constant shaking (220 rev min⁻¹).

Proteolytic activity and inhibition experiments

Alkaline and acid proteolytic activities were measured as before (Reichard et al., 2000) using azocasein (Sigma, St. Louis, MO) (Scharmann and Balke, 1974) and hemoglobin (Sigma) (Anson, 1938) as a substrate, respectively.

Phenylmethylsulfonylfluoride (PMSF, Serva, Heidelberg, Germany) was used according to standard protocols including the preincubation of enzyme-containing samples with the inhibitor at pH 7.0 (Barrett, 1994). Chymostatin or pepstatin (Peptide Institute, Osaka, Japan) were directly introduced into the substrate solutions of the proteinase assays (Barrett, 1994). Stock solutions of PMSF, chymostatin and pepstatin were prepared in ethanol, dimethylsulfoxide (DMSO) and methanol, respectively.

Purification of ALP2

For purification of ALP2, fungal cell wall debris in 50 mM Na-citrate buffer, pH 4.5, was prepared as described previously (Reichard et al., 2000), but at each washing step, pepstatin was added at a concentration of 1 µM. In addition, to prevent autolysis, all washing and the following purification steps were carried out at 4°C. To release ALP2 from the cell wall the debris was spun down and resuspended in 50 mM glycine-NaOH, pH 10.5, supplemented with 5 mM CaCl₂. Then, the ALP2-containing supernatant (30 ml) was separated from the debris by centrifugation (15,000 × g; 10 min), and ¼ volume of 1 M Tris-HCl, pH 7.5, was added. To optimize the binding conditions of ALP2 in hydrophobic exchange chromatography, the pH of the sample was adjusted to 8.0 by further addition of diluted HCl or NaOH. Subsequently, the sample was applied to a column containing 10 ml phenylbutylamine agarose (Sigma, St. Louis, MO), which had been thoroughly equilibrated with 0.2 M Tris-HCl, pH 8.0. After several rinses with the same buffer, the proteinase was eluted with 10 mM glycine-NaOH, pH 10.0. Proteinase-containing fractions (7 × 1 ml) were pooled and freeze dried. For SDS-PAGE and Western blot analysis, the freeze dried sample was redissolved in 200 µl H₂O.

Protein electrophoresis and sequencing of amino acids

Protein extracts were analyzed by SDS-PAGE with 12.5 % gels (Laemmli, 1970). For amino acid sequencing, the purified proteinase was blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) and stained with Coomassie® Brilliant blue R-250 (Merck, Darmstadt, Germany). N-terminal amino acid sequencing was accomplished by in situ Edman degradation using an Applied Biosystems Type 491 Protein Sequencer and the protocols of the manufacturer.

DNA manipulation

PCR reactions using degenerate oligonucleotide primers (A and B in Table 1) were performed with an Omnigene TR3 thermocycler (MWG-Biotech, Ebersberg, Germany) using the

Table 1. Primers used for PCR-amplifications.

A	5'-GAYCCACIGTIGAIWISIGC-3'
B	5'-TGIGGISWIGCCATISWIGTICC-3'
C	5'-GCCTACACATTGACACTGG-3'
D	5'-GTTGACTGCGTGCTTAGAGC-3'
E	5'-AAGGCATCAGAGCTGAGCAGC-3'
F	5'-TAGAAAGCGAAGACGGGCTCG-3'
G	5'-TATACTCCCGTTGCTCGTCGC-3'
H	5'-TGGAAAGAGTCCGATTAGCGG-3'

I = inosine; S = G/C; W = A/T; Y = C/T.

following conditions: Forty nanograms of genomic *A. fumigatus* DNA, 1.0 µl of each oligonucleotide at 50 µM and 5 µl of deoxynucleoside triphosphates each at 2 mM were dissolved in 50 µl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl₂, 0.01 % gelatin, 0.015 % Tween 20) also containing 2.5 units of Taq DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany). This reaction mixture was subjected to 5 cycles with 20 s at 95 °C, 20 s at 43 °C, 45 s at 72 °C (heating to 72 °C included a 3 s ramp per °C) followed by another 30 cycles with 20 s at 95 °C, 20 s at 53 °C and 50 s at 72 °C (Compton, 1990). The reaction was completed with an additional cycle at 72 °C for two minutes. Subsequently, the PCR sample was applied on a 1 % agarose gel and the band of interest was extracted with a QiaexII gel-extraction kit (Qiagen, Hilden, Germany). The DNA was then cloned into a plasmid, using the pCRII cloning kit (Invitrogen, San Diego, CA) according to the instructions of the supplier.

The gene encoding ALP2 was cloned using previously constructed (ZAP-cDNA and λ-Fix II-genomic libraries of *A. fumigatus* (Moser et al., 1992; Reichard et al., 2000). Screening of the cDNA library was performed using ³²P-labelled PCR products as a probe and standard protocols (Sambrook et al., 1989). The DNA was labelled using a random-primed DNA labelling kit (Roche, Mannheim, Germany) and [α-³²P]dATP. A pBluescript-II SK+ plasmid was excised from λ-ZAP bacteriophages using f1-helper bacteriophages (Short et al., 1988).

Screening of the genomic λ-Fix II-library was done with a digoxigenin-labelled PCR probe. The latter was produced by introducing digoxigenin-11-dUTP (Boehringer, Mannheim, Germany) and genomic DNA of *A. fumigatus* D141 into a PCR reaction. For detection of hybridizing plaques, peroxidase-labelled anti-digoxigenin-Fab fragments (Boehringer) were used according to the manufacturer's protocols. For Southern blots, standard protocols (Sambrook et al., 1989) and digoxigenin-labelled PCR probes using the same detection system as above were used. Agarose gel electrophoresis of restricted recombinant bacteriophage DNA and subcloning of genomic DNA fragments into the plasmid pMTL21 (Chambers et al., 1988) were performed according to standard protocols (Sambrook et al., 1989).

Double-stranded DNA, subcloned into pBluescript-II SK+ or pMTL21 was sequenced using the Applied Biosystems DNA Sequencer Model 373A and the protocols of the manufacturer.

A. fumigatus transformation

A. fumigatus was transformed according to the method described by Tilburn (Tilburn et al., 1983) and Paris (Paris, 1994). After overnight expression of the *HPH* gene, the transformants were incubated on agar, based on minimal medium containing 200 µg/ml hygromycin (Sigma), and selected after another 6 days of incubation at 20 °C. Initial transformant colonies were picked and again plated on hygromycin-containing agar. Growing colonies were screened by PCR for the replacement of the *ALP2* gene.

Analysis of the phenotype of the $\Delta alp2$ mutant

For comparison of vegetative growth of the $\Delta alp2$ mutant and its parental wild-type strain, agar plates based on mini-

mal medium (Cove, 1966) but varying in their carbon sources were used. Altogether, plates with nine different carbon sources (glucose, saccharose, mannose, mannitol, sorbitol, fructose, lactose, xylose, inositol) at a final concentration of 1 % were point-inoculated with approximately 10² conidia each, and the diameters of growing colonies at 37 °C were determined every 12 h for four days. Diameter values were plotted against the time, and the speed of colony growth was deduced for each carbon source and strain from the linear part of the curve.

To quantify sporulation, the surfaces of minimal medium agar deeps (3 ml in 15-ml conical centrifuge tubes) were inoculated with approximately 10⁴ freshly harvested conidia and incubated at 37 °C. The conidia from triplicate cultures were harvested daily in 0.1 % Tween 80 and counted in a haemocytometer. The morphology of conidiating structures was determined after three days of growth in slide cultures (Raper and Fennell, 1965), using minimal agar inoculated with approximately 20,000 conidia. Slides were stained in lactophenol-cotton blue (Raper and Fennell, 1965) and observed microscopically with a 40× objective (Carl Zeiss, Oberkochen, Germany).

Results

Purification of ALP2

Proteolytic activity against azocasein in the cell wall fraction of *A. fumigatus* was detectable over a broad pH range between 4.5 and 11.5. The protease which caused this activity was sensitive to PMSF and chymostatin, but not to pepstatin. It is called ALP2 in subsequent investigations.

ALP2 released from 10 g cell wall debris was purified on phenylbutylamine agarose, freeze dried and finally dissolved in 200 µl H₂O. The protein concentration was estimated to be approximately 3 mg/ml by absorption at 260 and 280 nm (Warburg and Christian, 1942) and by comparison with standards in SDS-PAGE. However, ALP2 did not migrate as a single band in SDS-PAGE, but rather as a low molecular weight smear (not shown), possibly due to strong autolytic activity of this enzyme. Introducing the inhibitor chymostatin into the sample prior to heating in SDS-sample buffer markedly improved the outcome and led to the occurrence of three major bands of approximately 34, 37 and 42 kDa (Fig. 1). Edman degradation of the 37 and 42 kDa band resulted in identical sequences (both: E-D-P-T-V-E-K-S-A-P) that showed four and five amino acid positions of identity with the N-terminus of *A. fumigatus* secreted subtilisin-related proteinase ALP1 (Jaton-Ogay et al., 1992; Reichard et al., 1990) and *A. niger* subtilisin-related PEPC (Frederick et al., 1993), respectively. The third, 34 kDa fragment was different in its N-terminus (T-I-D-T-G-I-N-V-D-H-V-D) but also showed partial

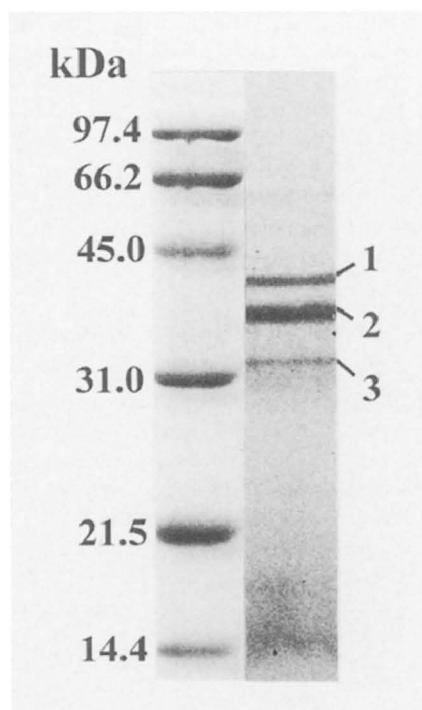


Fig. 1. Western blot of the purified ALP2 fraction on a PVDF membrane from 15% SDS-PAGE. The three major bands (1 = 42 kDa; 2 = 37 kDa; 3 = 34 kDa) of the same blot shown here were subjected to Edman degradation. Left lane: molecular weight marker (Biorad low).

identity with ALP1 (seven positions) and even complete identity with PEPC in a region approximately 50 amino acids downstream its N-terminus. Thus, it was likely that the 34 kDa band is derived from the same ALP2 molecule as the 37 and 42 kDa band and that a major part of the smear was derived from autolysis of the ALP2 enzyme.

Isolation of genomic and cDNA clones encoding ALP2

To approach the cDNA and genomic cloning of *ALP2*, a 750-bp PCR product of the gene was generated using degenerated oligonucleotide primers (A and B in Table 1). The primers were based on the N-terminal amino acid sequence obtained by Edman degradation of the mature proteinase and on the amino acid sequence region around the serine of the catalytic triad (Fig. 2) highly conserved in all serine proteinases of the subtilisin family (S8) (Rawlings and Barrett, 1994). The PCR product was cloned into a plasmid, using the pCRII cloning kit (Invitrogen, San Diego, CA) and then sequenced completely.

For screening the λ ZAP cDNA-library from *A. fumigatus*, the 750-bp insert was ^{32} P-labelled and 10 hybridizing plaques out of approximately 30,000 plaques screened were identified, subcloned into pBluescript II

SK+, and in part sequenced. Alternatively, the probe was digoxigenin-labelled and used to screen the genomic λ -FixII-library from *A. fumigatus*, which led to the isolation of 12 plaques out of which a common 4.8 kb BglII-fragment containing the *ALP2* gene was identified, subcloned into pMTL21 (Chambers et al., 1988) and restriction mapped (Fig. 3A). Sequence analysis of the genomic and cDNA did not reveal any difference in the coding regions between the two *A. fumigatus* strains used. The *ALP2* gene consists of 1623 bp encoding three exons and two introns. The deduced amino acid sequence revealed a 495 amino acid long subtilisin-related enzyme, with 49% identity with the vacuolar proteinase B of *S. cerevisiae* (Moehle et al., 1987) and 78% identity with PEPC, a serine proteinase of *A. niger* (Frederick et al., 1993). The identity with ALP1, the secreted subtilisin of *A. fumigatus* (Jaton-Ogay et al., 1992), is only 34%. An alignment of the proteinases is displayed in Fig. 2.

Based on the method of von Heijne (von Heijne, 1986), a 16 amino acid signal peptide was predicted, which is followed by a 120 amino acid long proregion located before the experimentally identified N-terminus. The mature protein theoretically consists of 359 amino acids with a calculated molecular mass of 37.5 kDa and matched the sequences of all the three 42, 37 and 34 kDa protein bands obtained by Edman degradation of purified ALP2. Further, the ALP2 sequence displays the catalytic triad (Asp/His/Ser) in the order common in all proteinases of the subtilisin family (A8) and the subtilisin-characteristic motifs around these active site residues (Fig. 2) (Rawlings and Barrett, 1994). In addition, there is a cysteine residue near the active site histidine (Cys 218 in Fig. 2) which is also present in the related PEPC of *A. niger* and PrB of *S. cerevisiae*, possibly indicating a requirement of ALP2 for thiol activation (Rawlings and Barrett, 1994).

The pI of mature ALP2 was calculated to be at pH 5.8, using the PCGENE program (IntelliGenetics, Geneva, Switzerland). Genomic DNA of *A. fumigatus* digested with various restriction enzymes revealed only single bands in high-stringency Southern blots using the 750-bp *ALP2* PCR product as a probe (not shown), suggesting a single copy of the gene in the *Aspergillus* genome. *ALP2* sequence data were submitted to EMBL database and are available with the accession No. Y13338 and AJ243145.

Replacement of the ALP2 gene

A transforming plasmid p Δ alp2-1 was designed as an ALP2 one-step gene replacement vector and was constructed using the plasmids pAN7-1 (Punt et al., 1987) and pMTL21 (Chambers et al., 1988) as shown in Fig. 3B. Additionally, a 4.8-kb cloned BglII *A. fumiga-*

tus DNA fragment containing the *ALP2* gene was used (Fig. 3A and B). In the final plasmid about 0.5 kb from the 5'-end of the *ALP2* gene and another 0.2 kb from its promoter region were missing. Instead, the plasmid contained the hygromycin resistance cassette of the pAN7-1. Prior to transformation, the p Δ *alp2*-1 plasmid was digested with BglII.

After transformation, 57 hygromycin-resistant colonies were isolated, using the p Δ *alp2*-1 plasmid (Fig. 3B and 4A). Screening for the desired replacement event (see Fig. 4A) was performed by PCR with only 12 of the transformants and a primer pair located within the theoretically replaced part of the *ALP2* gene (primer pair G + H in Table 1). Amplification of a 412-bp product indicated failure to delete *ALP2*. On the other hand, a negative PCR result indicated the replacement of *ALP2*. Six out of the 12 transformants were identified to carry the desired homologous integration and two of these mutants were then subjected to Southern blot analysis for further confirmation of the replacement events (Fig. 4).

Phenotypic analysis of the Δ *alp2* mutants

As expected, the Δ *alp2*-mutant lacked PMSF-sensitive activity against azocasein, while the pepstatin-sensitive proteolytic activity against hemoglobin, known to be caused by PEP2 (Reichard et al., 2000) was approximately at the same level as in the parental strain. In addition, all the six Δ *alp2* mutants identified by PCR exhibited a similar phenotype showing a color change of the mature colonies from greyish-green to white or light green (see Fig. 5A and B), independent from the medium chosen for growth. In contrast, the other six transformants with likely heterologously integrated p Δ *alp2*-1, did not show any difference in the phenotype compared to the parental strain. In total, 35 out of the 57 hygromycin-resistant transformants exhibited the color-changed phenotype of the identified Δ *alp2* disruptants.

The color change of the Δ *alp2*-mutant colonies was due to a significant decrease in formation of conidia. On minimal medium, formation of conidia in the mutant was about 20 % in comparison to the parental strain (Fig. 6A). The microscopic structure was characterized by an average smaller-sized conidiophore, and a reduction of the mean and median diameter of the mutant conidiophore vesicles to approximately half of those of the parental values (Table 2; Fig. 6B). However, both conidiophore morphologies were confluent: the typical wild-type conidiophore was also found in the mutant, and the typical mutant conidiophore was found in the wild type, although each typical morphology was only present in the other strain in comparatively low numbers. In addition, the speed of vegetative

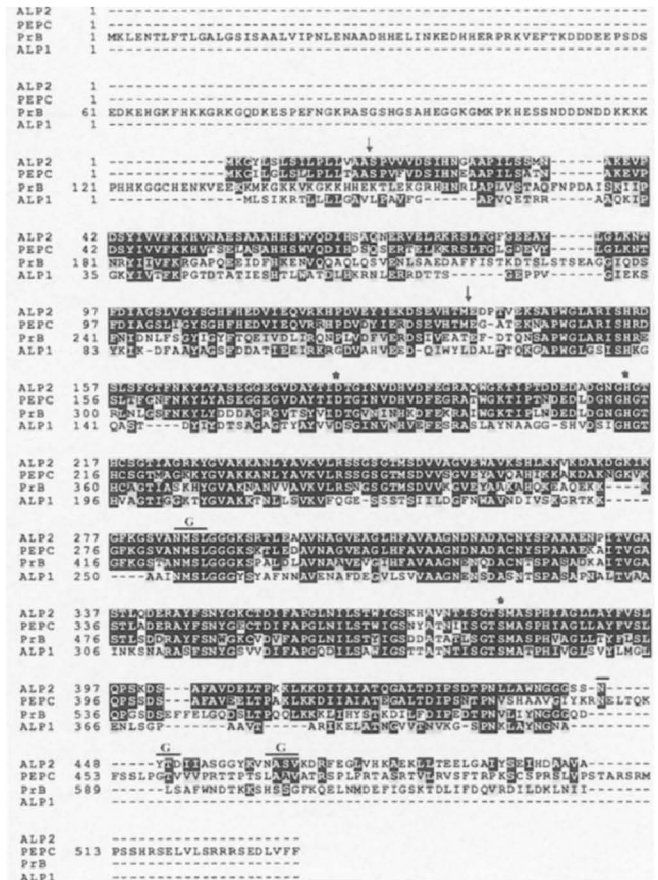


Fig. 2. Alignment of ALP2 against PEP2 from *A. niger* (Frederick et al., 1993), vacuolar proteinase PrB from *S. cerevisiae* (Moehle et al., 1987) and secreted ALP from *A. fumigatus* (Jaton-Ogay et al., 1992). Identical amino acids are black, homologous amino acids are gray shaded. The three putative N-glycosylation sites in ALP2 are designated with G. The three amino acids of the active triad of subtilisin-proteinases are labeled with an asterisk (*). Arrows (↓) indicate the cleavage site which separates the signal peptide from the proregion and the proregion from the mature enzyme, respectively.

growth of the Δ *alp2* mutant as measured by the time-dependent increase of colony diameters, was slightly reduced between 10–20 % of the values obtained for the wild type. This decreased growth was detected on all agar plates supplemented with different carbon sources and was also not dependent on the amount or source of nitrogen. The latter was examined using minimal medium-glucose agar containing normal or reduced levels of ammonium ions or no ammonium ions and instead 1 % gelatin (data not shown).

Discussion

We have purified an alkaline proteinase ALP2 which belongs to the subtilisin family from a cell wall fraction of *A. fumigatus*. Sequence analysis of ALP2 revealed a

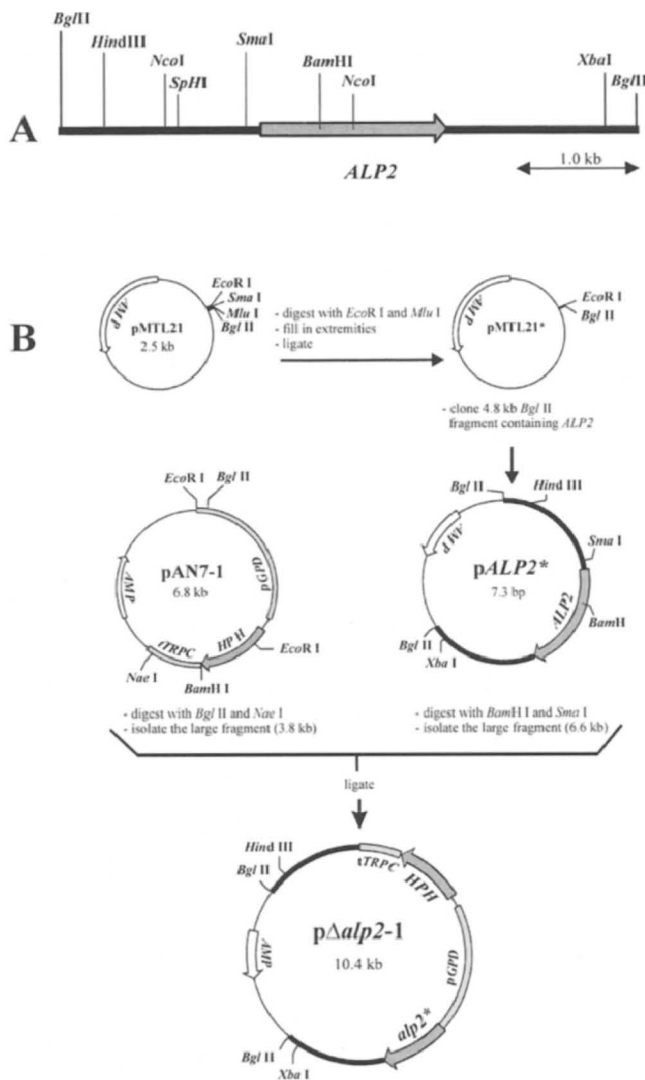


Fig. 3. **A:** Restriction map of the 4.8-kb BglII fragment containing the *ALP2* gene obtained from recombinant λ -Fix II bacteriophages. The gene consists of 1623 bp, including introns. **B:** Generation of plasmid *pΔalp2-1* on the basis of the restriction-mapped 4.8-kb *ALP2*-containing genomic fragment (Fig. 3A). Prior to transformation, *pΔalp2-1* was digested with BglII (insert in Fig. 4A).

close relationship to the major vacuolar proteinase PrB of *S. cerevisiae* (Moehle et al., 1987) and a proteinase of *A. niger* PEPC (Frederick et al., 1993). Although our data do not rule out the possibility that wall-associated ALP2 activity results from wall contamination during cell breakage, it is also very possible that ALP2 is a natural component not just of the vacuole but also of the wall. Indeed, overexpression of vacuolar proteinase PrA in *S. cerevisiae* as well as overexpression of the corresponding enzyme PEP2 of *A. fumigatus* in the yeast *Pichia pastoris*, led to the occurrence of the enzymes in the fungal culture supernatants (Reichard et al., 2000; Rothman et al., 1986). These findings demonstrate

that under certain circumstances sorting of vacuolar proteinases which takes place in the late Golgi compartment (for review see (Jones et al., 1997)) is not necessarily a strictly uni-directed event. Thus, additional extracellular sorting combined with a high affinity of ALP2 to the cell wall, is a reasonable explanation for our finding of the enzyme in the wall debris. However, studies in different developmental stages of *A. fumigatus* are in progress to examine the localization of ALP2 in the cell more closely.

Altogether, three ALP2 products of 42, 37 and 34 kDa were detected on SDS-PAGE from a cell wall preparation. The 42- and 37-kDa ALP2 products (Fig. 1) showed identical N-termini. It is likely that the size difference of these ALP2 products is due to a processing of the C-terminal part of the protein as it is known for *S. cerevisiae* PrB. Here, in addition to the removal of the N-terminus propeptide, two additional cleavages at the C-terminus take place which together remove about 60 additional amino acids (Moehle et al., 1989). Assuming a similar process in *A. fumigatus*, the sequence length of mature ALP2 might be not the calculated 359 amino acids but only about 300 amino acids which corresponds to the 37-kDa ALP2 product visualized on SDS-PAGE. The 34-kDa band (Fig. 1) however, most likely reflects a degradation product of ALP2.

The *Δalp2* mutants were largely impaired in their production of asexual spores and also exhibited a slightly reduced speed in their vegetative growth. Comparing the *Δalp2* mutant and parental strain colonies microscopically, no specific block in the genesis of the conidiophores, phialides, or conidia was observed in the mutants. Conidium and phialide morphologies were similar in both strains. It was not practicable to determine if any differences in overall conidiophore densities in the two strains might correlate with the mutant's reduction in conidium production. However, the average size of the conidiophore was reduced and, in particular, the mean and median diameter of the mutant conidiophore vesicles were approximately half of those of the parental values (Table 2; Fig. 6B). Assuming phialide distribution over one half of the vesicle surface, an approximately 75 % reduction on phialide density is calculated per conidiophore. On the other hand, reduction of vegetative growth of the *Δalp2* mutant was between 10 and 20 % and may contribute to its impaired production of conidia. However, as shown in Figure 6A, time-dependent increase in spore numbers of mutant and wild-type cultures does not reflect a simple delay of sporulation in the mutants. Instead, the number of conidia of the mutant reaches a nearly time-independent maximum of approximately 20 % of that of the wild-type strain. Thus, it is likely that almost the entire reduction in conidium produc-

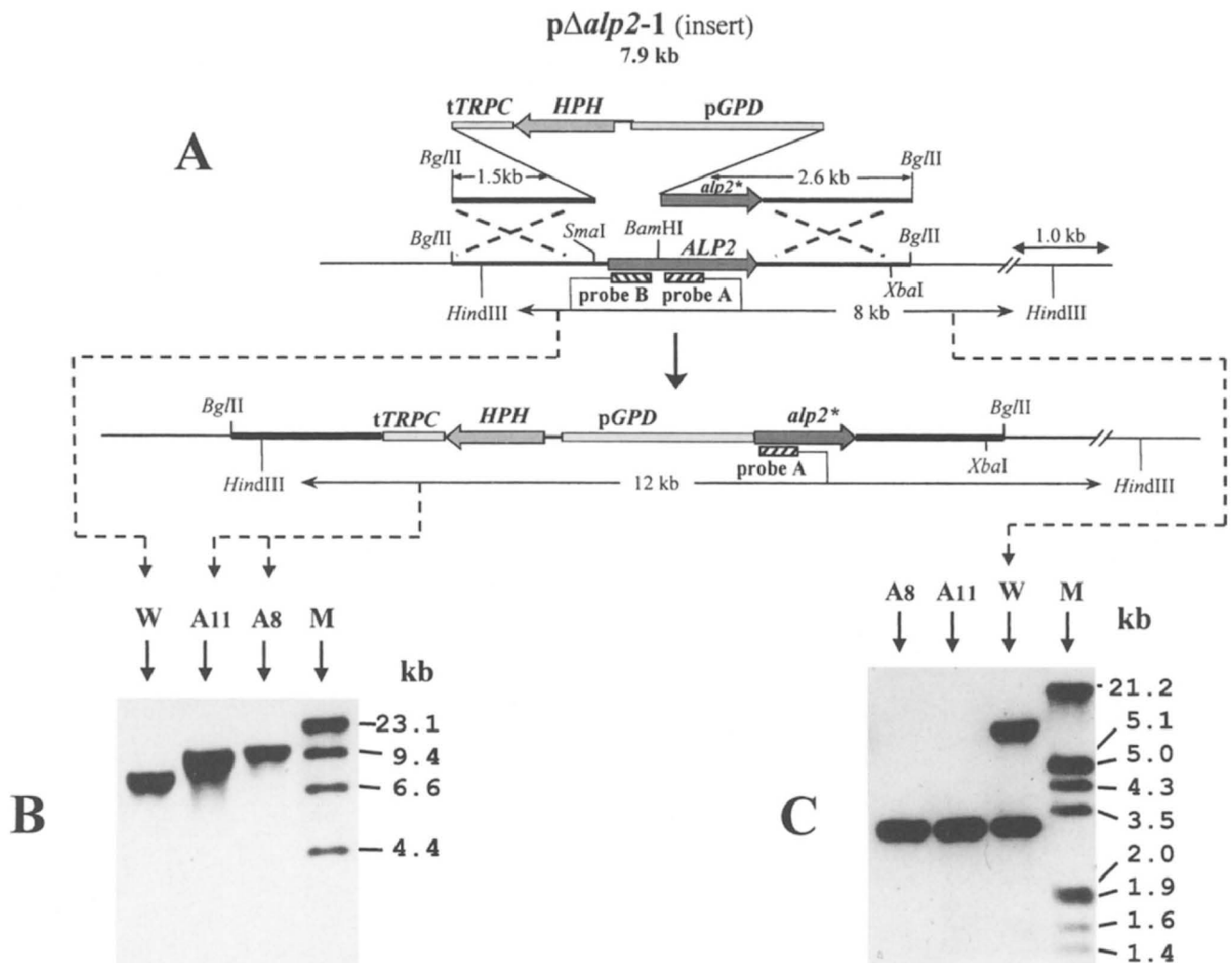


Fig. 4. **A:** Predicted cross-over event using *Bgl*II-digested *pΔalp2-1* (see Fig. 3B). **B:** Southern blot analysis of *ALP2*-deletion strains (A8 and A11) and their parental wild-type strain (W) using a probe hybridizing outside of the deleted region (note the location of probe A in Fig. 4A). Chromosomal DNA was subjected to electrophoresis after *Hind*III digestion. In the wild-type strain the probe hybridized with an approximately 8.0-kb genomic fragment. Following homologous integration of the transforming DNA, the predicted size of the hybridizing *Hind*III fragment is 12 kb, which is in agreement with the size of the hybridizing bands in both mutants. In addition, the single bands in the mutants provide evidence for a single integration event of the transforming DNA into the fungal genome. The 591-bp probe A used was generated by PCR on *A. fumigatus* D141 DNA and the primers displayed in Table 1 (primer C + D). M = digoxigenin-labeled DNA size marker (Roche). **C:** Southern blot analysis of *ALP2*-deletion strains (A8 and A11) and their parental wild-type strain (W) using a probe hybridizing with the deleted region (note the location of probe B in Fig. 4A). The Southern blot was hybridized with probe B which detected the 8.0-kb *ALP2* gene-containing fragment in the wild type and a second probe C (not displayed in Fig. 4A) detecting another known proteinase gene of *Aspergillus* on a 3.0-kb fragment as a control (PEP2; EMBL Accession No. Y15744 and AJ132504). Note that both of the mutants and the wild-type strain showed hybridization with the control probe C on the 3.0-kb fragment, whereas probe B only hybridized with the wild type. Thus, there is evidence that as a result of homologous integration of the transforming DNA the *ALP2* gene is missing in the genome of the two mutants. The two probes used were generated by PCRs on *A. fumigatus* D141 DNA and primers displayed in Table 1 (primer G + H for 412 bp-sized probe B; primer E + F for 479 bp-sized probe C). M = digoxigenin-labeled DNA size marker (Boehringer).

tion in the mutant can be attributed to the smaller size of the conidiophore vesicle.

The phenotype of the *Δalp2* mutants, which does not show a block but rather a starvation pattern of conidiophore development, would be in agreement with the assumption that *ALP2* like its homologue *PrB* is also a vacuolar proteinase responsible for the general supply of amino acids needed for the synthesis of de-

velopmental specific proteins. Since the *Δalp2* mutants showed comparable phenotypes on Sabouraud and on minimal agar (Fig. 5), the source of substrate proteins indeed should be rather intracellular (e.g. unneeded structural or metabolic proteins) than extracellular. Interestingly, knock-out mutants of *PEP2* in *A. fumigatus*, which is likely to be the corresponding enzyme of vacuolar *PrA* in *S. cerevisiae*, did not show a develop-

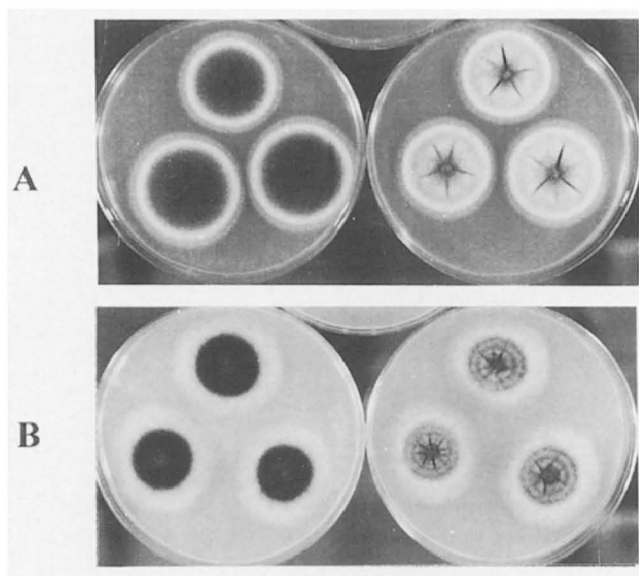


Fig. 5. **A:** Parental strain *A. fumigatus* D141 (left) and $\Delta alp2$ mutant A8 (right) grown on Sabouraud agar plates for three days at 37°C. The $\Delta alp2$ mutant showed significantly reduced sporulation seen here as a typical white colony. Note that a smaller central zone in the $\Delta alp2$ mutant is pigmented. **B:** Parental strain *A. fumigatus* D141 (left) and $\Delta alp2$ mutant A8 (right) grown for 4 days at 37°C on minimal medium containing no proteins, peptides or amino acids (Cove, 1966). Note again the reduced pigmentation in the $\Delta alp2$ mutant.

mental phenotype (Reichard et al., 2000). Thus, in contrast to yeasts where functional PrA is a key developmental enzyme normally required for PrB activity (Jones et al., 1997), in *Aspergillus* the PrB homologue ALP2 seems to be the more independent and crucial protease for development.

Another aspect that should be taken into consideration derives from the observation that PrB in *S. cerevisiae* is not merely a digesting enzyme of the vacuole but also a multiple hydrolase-activating enzyme (for review see (Jones et al., 1997)). Therefore, the effects of its homologue ALP2 on conidiophore and conidial development may also reflect the activation of other proteins involved in this process. A good example of such an important processing modulator proteinase is the calpain-like cysteine proteinase (CPL1) in yeast. This enzyme has a unique role in signal transduction via an indirect activation of RIM101, a key transcription factor involved in sexual sporulation (Futai et al., 1999). Albeit sexual sporulation in *S. cerevisiae* genetically is distinct from asexual sporulation in *Aspergillus*, the possibility exists that ALP2 may perform a similar function involving activation of a corresponding transcription factor, in addition to its suspected vacuolar functions.

Although there is a slight difference in molecular mass (37 versus 34 kDa in SDS-PAGE) and a similar amino acid substitution in one position of their N-termini, ALP2 is likely to be identical with the major allergen of *A. fumigatus* in patients suffering from allergic asthma (Shen et al., 1999). We have found ALP2 to be a strong autolytic enzyme, and observed mass variation of corresponding bands particularly when no proteinase inhibitors were added to the samples. In addition, even though one amino acid among the nine identified at the N-terminus of ALP2 differs from that of the allergen (Shen et al., 1999), this should be interpreted as supporting identity between the two rather than as indicating that they are different members of a common family. N-terminal variation is high even among related fungal subtilisins (see Fig. 2), and the

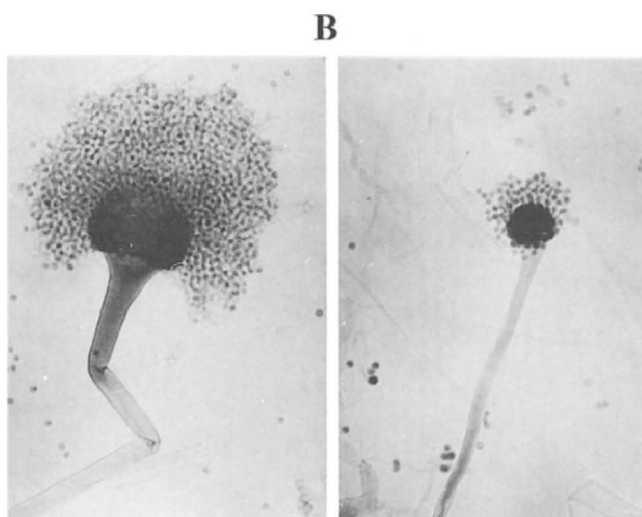
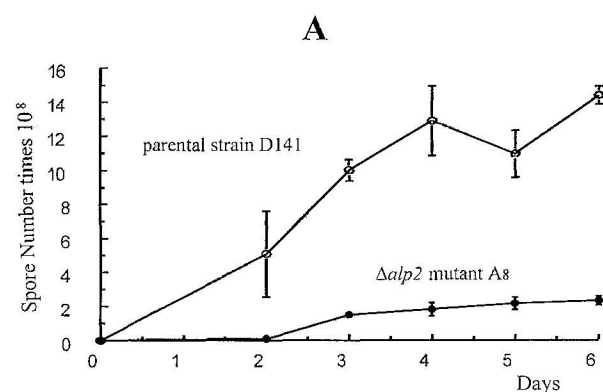


Fig. 6. **A:** Time course of conidium production in parental strain D141 and mutant strain A8 of *A. fumigatus*. Vertical error bars equal plus or minus one standard deviation. **B:** Typical conidiophore morphology of parental strain D141 (left) and mutant strain A8 (right). Note that the sizes of conidia are equal in both strains. Magnification 700-fold.

Table 2. The effect of ALP2 deletion upon conidiophore vesicle morphology.

Strain	Mean vesicle diameter (µm) (c)	Range (µm)	Median vesicle diameter (µm)	Median vesicle diameter (µm ²) (e)
Parental (a=59)	21.4 +/- 5.9 (b)	8–32	22	760
A8 (a=60)	12.1 +/- 5.3 (b)	4–28	11 (50%) (d)	190 (25%) (d)

(a) Number of observations; (b) Standard deviation; (c) Different at the 0.001 level of significance; (d) Percent of the parental value; (e) One-half the area of an idealized sphere.

small difference seen between these two sequences almost certainly results merely from strain variation.

In fact, being a major allergen supports a cell-wall or surface localization of ALP2, because allergic asthma usually is characterized by an immediate response of pulmonary mast cells which release mediators of hypersensitivity upon direct contact with the inhaled allergen. The potential crucial role of ALP2 in patients with allergic asthma together with its significant contribution to efficient conidia production in the fungus should now give reason for further detailed medical and biological investigations of this class of cell wall-associated enzymes in pathogenic fungi.

Acknowledgement. We are indebted to Ms Birgit Riemschneider and Ms Meike Schaffrinski for technical assistance at various stages of this investigation.

References

Anson, M. L.: The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *J. Gen. Physiol.* 22, 79–91 (1938).
 Barrett, A. J.: Classification of peptidases. *Methods Enzymol.* 244, 1–15 (1994).
 Chambers, S. P., Prior, S. E., Barstow, D. A., Minton, N. P.: The pMTL nic- cloning vectors. I. Improved pUC poly-linker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* 68, 139–149 (1988).
 Chow, L. P., Liu, S. L., Yu, C. J., Liao, H. K., Tsai, J. J., Tang, T. K.: Identification and expression of an allergen asp f 13 from *Aspergillus fumigatus* and epitope mapping using human IgE antibodies and rabbit polyclonal antibodies. *Biochem. J.* 346, 423–431 (2000).
 Cockrill, B. A., Hales, C. A.: Allergic bronchopulmonary aspergillosis. *Annu. Rev. Med.* 50, 303–316 (1999).
 Compton, T.: Degenerate primers for DNA amplification. In: *PCR Protocols* (M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White, eds.), pp. 39–45. Academic Press, San Diego 1990.
 Cove, D. J.: The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochim. Biophys. Acta* 113, 51–56 (1966).

Cramer, R.: Recombinant *Aspergillus fumigatus* allergens: from the nucleotide sequences to clinical applications. *Int. Arch. Allergy Immunol.* 115, 99–114 (1998).
 Cramer, R.: Epidemiology and molecular basis of the involvement of *Aspergillus fumigatus* in allergic diseases. *Contrib. Microbiol.* 2, 44–56 (1999).
 Cramer, R., Blaser, K.: Cloning *Aspergillus fumigatus* allergens by the pJuFo filamentous phage display system. *Int. Arch. Allergy Immunol.* 110, 41–45 (1996).
 Denning, D. W.: Invasive aspergillosis. *Clin. Infect. Dis.* 26, 781–805 (1998).
 Frederick, G. D., Rombouts, P., Buxton, F. P.: Cloning and characterisation of *pepC*, a gene encoding a serine protease from *Aspergillus niger*. *Gene* 125, 57–64 (1993).
 Futai, E., Maeda, T., Sorimachi, H., Kitamoto, K., Ishiura, S., Suzuki, K.: The protease activity of a calpain-like cysteine protease in *Saccharomyces cerevisiae* is required for alkaline adaptation and sporulation. *Mol. Gen. Genet.* 260, 559–568 (1999).
 Jatton-Ogay, K., Paris, S., Huerre, M., Quadroni, M., Falchetto, R., Togni, G., Latgé, J. P., Monod, M.: Cloning and disruption of the gene encoding an extracellular metalloprotease of *Aspergillus fumigatus*. *Mol. Microbiol.* 14, 917–928 (1994).
 Jatton-Ogay, K., Suter, M., Cramer, R., Falchetto, R., Fatihi, A., Monod, M.: Nucleotide sequence of a genomic and a cDNA clone encoding an extracellular alkaline protease of *Aspergillus fumigatus*. *FEMS Microbiol. Lett.* 71, 163–168 (1992).
 Jones, E. W., Webb, G. C., Hiller, M. A.: Biogenesis and Function of the Yeast Vacuole. In: *The Molecular and Cellular Biology of the Yeast Saccharomyces: Cell Cycle and Cell Biology* (J. R. Pringle, J. R. Broach and E. W. Jones, eds.), vol. 3, pp. 363–470. Cold Spring Harbor Laboratory Press, New York 1997.
 Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685 (1970).
 Latgé, J. P.: *Aspergillus fumigatus* and aspergillosis. *Clin. Microbiol. Rev.* 12, 310–350 (1999).
 Lee, J. D., Kolattukudy, P. E.: Molecular cloning of the cDNA and gene for an elastinolytic aspartic proteinase from *Aspergillus fumigatus* and evidence of its secretion by the fungus during invasion of the host lung. *Infect. Immun.* 63, 3796–3803 (1995).
 Moehle, C. M., Aynardi, M. W., Kolodny, M. R., Park, F. J., Jones, E. W.: Protease B of *Saccharomyces cerevisiae*: isolation and regulation of the *PRB1* structural gene. *Genetics* 115, 255–263 (1987).
 Moehle, C. M., Dixon, C. K., Jones, E. W.: Processing pathway for protease B of *Saccharomyces cerevisiae*. *J. Cell Biol.* 108, 309–325 (1989).
 Monod, M., Paris, S., Sanglard, D., Jatton-Ogay, K., Bille, J., Latgé, J. P.: Isolation and characterization of a secreted metalloprotease of *Aspergillus fumigatus*. *Infect. Immun.* 61, 4099–4104 (1993).
 Monod, M., Togni, G., Rahalison, L., Frenk, E.: Isolation and characterisation of an extracellular alkaline protease of *Aspergillus fumigatus*. *J. Med. Microbiol.* 35, 23–28 (1991).

- Moser, M., Cramer, R., Menz, G., Schneider, T., Dudler, T., Virchow, C., Gmachl, M., Blaser, K., Suter, M.: Cloning and expression of recombinant *Aspergillus fumigatus* allergen I/a (rAsp f I/a) with IgE binding and type I skin test activity. *J. Immunol.* 149, 454–460 (1992).
- Paris, S.: Isolation of protease negative mutants of *Aspergillus fumigatus* by insertion of a disrupted gene. In: *Molecular Biology of Pathogenic fungi. A Laboratory Manual* (B. Maresca and G. S. Kobayashi, eds.), pp. 49–55. Telos Press, New York 1994.
- Punt, S. J., Oliver, P. O., Dingemans, M. A., Pouwels, P. H., van den Hondel, C. A. M. J. J.: Transformation of *Aspergillus* based on hygromycin B resistance marker from *Escherichia coli*. *Gene* 56, 117–124 (1987).
- Raper, K. B., Fennell, D. I.: *The Genus Aspergillus*. The Williams & Wilkins Co., Baltimore 1965.
- Rawlings, N. D., Barrett, A. J.: Families of serine peptidases. *Methods Enzymol.* 244, 19–61 (1994).
- Reichard, U., Büttner, S., Eiffert, H., Staib, F., Röchel, R.: Purification and characterisation of an extracellular serine proteinase from *Aspergillus fumigatus* and its detection in tissue. *J. Med. Microbiol.* 33, 243–251 (1990).
- Reichard, U., Cole, G. T., Röchel, R., Monod, M.: Molecular cloning and targeted deletion of *PEP2* which encodes a novel aspartic proteinase from *Aspergillus fumigatus*. *Int. J. Med. Microbiol.* 290, 85–96 (2000).
- Reichard, U., Eiffert, H., Röchel, R.: Purification and characterization of an extracellular aspartic proteinase from *Aspergillus fumigatus*. *J. Med. Vet. Mycol.* 32, 427–436 (1994).
- Reichard, U., Monod, M., Odds, F., Röchel, R.: Virulence of an aspergillopepsin-deficient mutant of *Aspergillus fumigatus* and evidence for another aspartic proteinase linked to the fungal cell wall. *J. Med. Vet. Mycol.* 35, 189–196 (1997).
- Rothman, J. H., Hunter, C. P., Valls, L. A., Stevens, T. H.: Overproduction-induced mislocalization of a yeast vacuolar protein allows isolation of its structural gene. *Proc. Natl. Acad. Sci. USA* 83, 3248–3252 (1986).
- Röchel, R., Reichard, U.: Pathogenesis and clinical presentation of aspergillosis. In: *Aspergillus fumigatus. Biology, Clinical Aspects and Molecular Approaches to Pathogenicity* (A. Brakhage, B. Jahn and A. Schmidt, eds.), vol. 2, pp. 21–43. Karger AG, Basel 1999.
- Sambrook, J., Fritsch, E. F., Maniatis, T.: *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York 1989.
- Scharmann, W., Balke, E.: Studies on a protease (elastase) from *Pseudomonas aeruginosa*, I: formation and purification of the protease. *Hoppe Seyler's Z. Physiol. Chem.* 355, 443–450 (1974).
- Shen, H. D., Tam, M. F., Chou, H., Han, S. H.: The importance of serine proteinases as aeroallergens associated with asthma. *Int. Arch. Allergy Immunol.* 119, 259–264 (1999).
- Short, J. M., Fernandez, J. M., Sorge, J. A., Huse, W. D.: Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. *Nucleic Acids Res.* 16, 7583–7600 (1988).
- Tang, C. M., Cohen, J., Krausz, T., Van Noorden, S., Holden, D. W.: The alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. *Infect. Immun.* 61, 1650–1656 (1993).
- Tilburn, J., Scazzocchio, C., Taylor, G. G., Zabicky-Zissman, J. H., Lockington, R. A., Davies, R. W.: Transformation by integration in *Aspergillus nidulans*. *Gene* 26, 205–221 (1983).
- von Heijne, G.: A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14, 4683–4690 (1986).
- Warburg, O., Christian, W.: Isolierung und Kristallisation des Gärungsfermentes Enolase. *Biochemische Zeitschrift* 310, 384–421 (1942).