

Recombinant Expression and Antigenic Properties of a 32-Kilodalton Extracellular Alkaline Protease, Representing a Possible Virulence Factor from *Aspergillus fumigatus*

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A 32-kDa nonglycosylated alkaline protease (EC 3.4.1.14) with elastolytic activity, secreted by the opportunistic pathogen *Aspergillus fumigatus* ATCC 42202, is suggested to be a virulence factor of this fungus. The enzyme is a serine protease of the subtilisin family, and its cDNA nucleotide sequence has recently been reported. We have cloned the cDNA encoding the mature protease into a high-level *Escherichia coli* expression plasmid and produced the recombinant protease as a fusion protein with a six-adjacent-histidine affinity tag at the carboxy terminus. Subsequently, the recombinant protease was purified to homogeneity, with affinity chromatography yielding 30 to 40 mg of recombinant protease per liter of *E. coli* culture. Refolded recombinant protease, in comparison with native protease, demonstrated weak enzymatic activity but similar immunological characteristics as analyzed by antigen-specific enzyme-linked immunosorbent assay (ELISA), competition ELISA, and immunoblotting assays. To assess the allergenic potential of the protease, sera from patients with allergic bronchopulmonary aspergillosis and sera from healthy control individuals were analyzed by ELISA and immunoblotting techniques. Sera from patients with allergic bronchopulmonary aspergillosis did not have protease-specific immunoglobulin E (IgE) antibodies and, remarkably, did not show significantly elevated protease-specific IgG antibody levels compared with those in sera from healthy control individuals. This suggests that the alkaline protease from *A. fumigatus* does not elicit IgE antibodies and has weak immunogenicity, a property which may explain fungus persistence in allergic individuals.

Aspergillus fumigatus, found ubiquitously in nature, is an opportunistic airborne pathogen affecting humans (2). It accounts for a variety of respiratory disorders, such as hypersensitivity pneumonitis, immunoglobulin E (IgE)-mediated asthma, allergic bronchopulmonary aspergillosis (ABPA) (3, 9, 22), and aspergilloma, as well as severe invasive infections (18, 36) in immunocompromised patients undergoing chemotherapy or after organ transplantation. Further, *A. fumigatus* infections are reported in a small proportion of human immunodeficiency virus-infected patients (6, 23). The most severe *A. fumigatus*-induced respiratory disease in allergic immunocompetent patients is ABPA. Clinically, patients with ABPA manifest bronchial asthma with transient pulmonary infiltrates that finally lead to lung fibrosis (3, 9, 22). Therefore, precise diagnosis and adequate treatment are required to prevent progressive lung impairment.

Since well-characterized and homogenic fungal allergens are lacking (14, 44), the immunopathologic mechanisms leading to *A. fumigatus* allergic diseases are difficult to elucidate. The availability of pure immunoreactive antigens or allergens may improve the diagnosis and/or the immunotherapy of *A. fumigatus* allergic diseases (34) and may lead to a better understanding of allergic reactions underlying immunopathological complications, like IgE-mediated asthma or ABPA. To circumvent problems related to extract standardization, pure recombinant allergens from *A. fumigatus* have been produced (17, 27). Clinical studies have shown the advantages of the recombinant *A. fumigatus* allergen I/a in the diagnosis of *A. fumigatus* allergy (26, 28).

Various proteases from *A. fumigatus* have been biochemically purified and characterized (8, 21, 25, 30). Proteases seem to play a major role in the pathogenesis of *A. fumigatus* as well as of dental plaque bacteria (39), *Pseudomonas aeruginosa* (4), and *Candida albicans* (20). The elastolytic activity of *A. fumigatus* proteases may enhance invasion of tissue and cell detachment (30, 32). Furthermore, Kothary et al. (16) correlated elastase production by *A. fumigatus* with its ability to cause pulmonary invasive aspergillosis in immunocompromised mice. In addition, in clinical *A. fumigatus* isolates elastase production has been shown to be an important virulence factor in invasive aspergillosis (15, 31).

We focused our attention on a secreted alkaline protease from *A. fumigatus* reported to be a serine protease of the subtilisin family possessing elastolytic activity (25). Recently, we have nucleotide sequenced a genomic and a cDNA clone encoding the mature 282-amino-acid-long protease with a 121-amino-acid prepro-leader sequence (13). In the present report, we describe the expression of the mature form of the protease in *Escherichia coli* and its purification to homogeneity by a single affinity chromatography step. Focusing on the role of the alkaline protease in the pathogenesis of *A. fumigatus*-associated diseases, we analyzed the immunogenic properties of the protease sera from patients suffering from allergic respiratory diseases.

MATERIALS AND METHODS

Subjects. Sera from 19 patients with ABPA and 6 healthy control individuals were used. Patients with ABPA had asthma and fulfilled at least six of the seven criteria proposed by Rosenberg et al. (33) and Patterson et al. (29): blood eosinophilia of $>1,000/\text{mm}^3$, immediate cutaneous reactivity to *A.*

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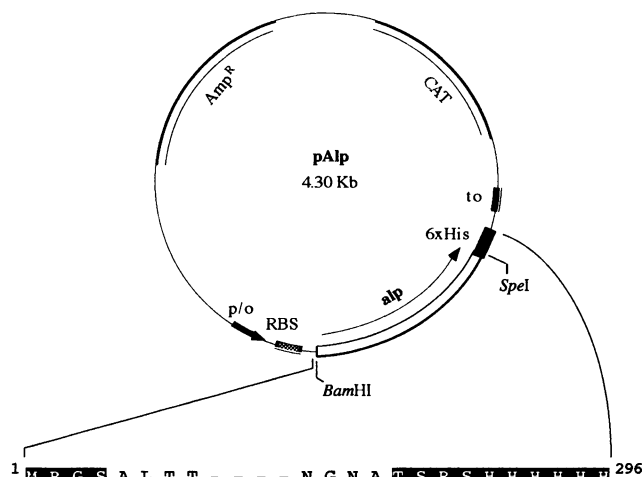


FIG. 1. Construction of the expression vector pAlp. The cDNA encoding the mature protease (□) was inserted into the *Bam*HI and *Spe*I restriction sites of plasmid pAlp. The entire sequence coding for the fusion extensions (white letters on black) and nucleotides of the cDNA encoding the protease (black letters on white) are depicted. p/o, promoter/operator region; RBS, synthetic ribosome binding site; 6xHis, six-histidine fusion tag; to, transcriptional terminator of phage lambda; Amp^R, ampicillin resistance gene; CAT, chloramphenicol acetyltransferase gene.

fumigatus, precipitating antibodies to *A. fumigatus*, elevated total serum IgE concentration, history of infiltrates on chest X ray, central bronchiectasis, and elevated serum IgE and IgG specific to *A. fumigatus*. The healthy control individuals had no history of allergy or asthma and had normal serum IgE levels.

Strains, culture conditions, and plasmids. *A. fumigatus* ATCC 42202 was isolated from sputum of a patient with aspergilloma (19) and used for construction of a cDNA library (27). *E. coli* M15 with repressor plasmid pREP4 (41) was used for high-level expression and was grown on Luria-Bertani (LB) plates, LB medium, or 2× YT medium, all containing 100 µg of ampicillin and 25 µg of kanamycin per ml (35). Plasmid pDS56 was kindly provided by D. Stüber (Hoffmann-La Roche, Ltd., Basel, Switzerland) and modified by introduction of an *Spe*I restriction site located 5' to the histidine tag by using standard protocols (35) (Fig. 1).

Enzymes and reagents. DNA-modifying enzymes and restriction endonucleases were obtained from Boehringer (Mannheim, Germany) and used as recommended by the supplier. Chemicals and reagents were obtained from Sigma (St. Louis, Mo.), Merck (Darmstadt, Germany), or Fluka (Buchs, Switzerland) if not otherwise specified. *Taq* DNA polymerase used for PCR was from Anawa Trading (Wangen, Switzerland). Biochemically purified native alkaline proteases from *A. fumigatus* (CHUV 192-88) and *Aspergillus oryzae* were kindly provided by M. Monod (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland).

Antibodies. The following antibodies were used in enzyme-linked immunosorbent assay (ELISA) and/or immunoblotting and diluted as recommended by the manufacturer: alkaline phosphatase-conjugated goat anti-human IgG (γ chains) from TAGO (Burlingame, Calif.), sheep anti-human IgE (ε chain) from The Binding Site (Birmingham, England), and affinity-purified sheep IgG Fab fragments to digoxigenin conjugated with alkaline phosphatase from Boehringer.

Digoxigenin labeling of rabbit antiserum. Polyclonal sera from New Zealand White rabbits immunized with purified native protease from *A. fumigatus* were kindly supplied by M. Monod (Centre Hospitalier Universitaire Vaudois) (25). The rabbit antisera were precipitated with ammonium sulfate, and then the immunoglobulins were dialyzed against 0.1 M NaHCO₃, pH 8.0, for 1 day and diluted to a final concentration of 1 mg/ml. Labeling was performed with the digoxigenin antibody labeling kit from Boehringer. The reaction was kept at room temperature for 2 h. Unbound digoxigenin was removed by dialysis against 0.1 M NaHCO₃ for 2 days with a molecular porous membrane with a 3.5-kDa cutoff (SPECTRA/POR, Houston, Tex.).

Construction of expression vector pAlp. The cDNA sequence encoding the mature protease was isolated by PCR from the full-length cDNA clone (13). Therefore, the N primer and the C primer designed to anneal to the appropriate cDNA sequence contained flanking *Bam*HI and *Spe*I restriction sites for unidirectional cloning (Table 1). As the DNA template for PCR, we used the previously described and nucleotide-sequenced cDNA clone inserted into plasmid pMTL21 (13). PCR was performed in a volume of 50 µl with a Techne PHC-2 thermocycler (Brouwer, Luzern, Switzerland). The sample contained the appropriate buffer, 200 µM each deoxynucleotide triphosphate, 0.5 µM each N primer and C primer, 2 ng of DNA template, and 1 U of *Taq* DNA polymerase. Samples were subjected to two cycles of amplification consisting of a 1-min denaturation at 95°C, annealing for 1 min at 50°C, and 1 min of extension at 72°C, followed by 30 cycles under identical conditions except that the annealing was done at 55°C. The 862-bp-long PCR product was purified by using the double GeneClean procedure (Bio 101, Inc., La Jolla, Calif.), digested with *Bam*HI and *Spe*I, and ligated into plasmid pDS56 by standard protocols (35). Vector pAlp (Fig. 1) was used to transform competent *E. coli* M15 cells by using a Gene Pulser (Bio-Rad, Richmond, Calif.), and transformants were selected on LB plates with appropriate antibiotics. Plasmid DNA preparation was performed with the plasmid kit from QIAGEN, Inc. (Kontron Instruments AG, Schlieren, Switzerland).

Production, purification, and refolding. Expression of the recombinant protease was induced in a mid-logarithmic-phase *E. coli* culture (1 liter) by addition of 2 mM isopropyl-β-D-thiogalactopyranoside (41). After an additional 4 h of incubation, cells were pelleted and lysed overnight in 100 ml of 6 M guanidine hydrochloride, pH 8.0. Cleared lysate was applied to a column packed with 12 ml of Ni²⁺-chelate affinity resin (QIAGEN) at a flow rate of 0.7 ml/min at room temperature. The recombinant alkaline protease was eluted with an 8 M

TABLE 1. PCR primers^a

Primer	Nucleotide sequence	Encoded protease amino acids
N primer	5'CGGGATCCGCCTTGACCACTCAAAGGG3'	Ala Leu Thr Thr Gln Lys
C primer	5'GGACTAGTAGCATTGCCATTGTAGG3'	Tyr Asn Gly Asn Ala

^a N primer, amino-terminal end of the protease (12) with introduced *Bam*HI restriction site (underlined); C primer, carboxy-terminal end of the protease (12) with engineered *Spe*I restriction site (underlined).

urea pH step gradient (10) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For renaturation, the recombinant protease was diluted 1:100 with H₂O, resulting in a urea concentration of 80 mM. Renatured recombinant protease was concentrated by ultrafiltration with a Centricon 10 unit (Amicon Corp., Danvers, Mass.), and protein concentration was determined with the Bio-Rad protein assay with bovine serum albumin as the standard. The refolded protease was stored at 4°C for 1 week prior to testing for enzymatic activity.

Enzymatic activity assay. The enzymatic activity was assayed with protease substrate gel tablets (Bio-Rad). Briefly, two casein substrate gel tablets were hydrated in 15 ml of H₂O for 20 min at room temperature. After vortexing, the mixture was heated for 5 min in a boiling water bath and immediately dispersed onto a plastic diffusion plate. After setting of the gel, sample wells were punched in the gel, in each of which a 15- μ l sample was loaded. The plates were incubated at 24°C for 20 h, and the diameter of the rings was measured.

Protein electrophoresis and immunoblotting. SDS-12% PAGE gels (2 to 5 μ g of protein per track) were run under denaturing and reducing conditions (35) by using a Midget Electrophoresis Unit (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Staining of gels with Coomassie brilliant blue G250 (Merck) was as described elsewhere (35).

For immunoblotting, proteins (2 to 5 μ g per track) were separated on an SDS-12% PAGE gel and transferred (35) to a Hybond-ECL nitrocellulose membrane (Amersham, Amersham, Buckinghamshire, England) by using a Multiphor II Electrophoretic Transfer Unit (Pharmacia) at 60 mA for 2 h. The membrane was blocked at room temperature for 1 h with phosphate-buffered saline solution (PBS), pH 7.4, containing 5% (wt/vol) nonfat dry milk powder and 0.5% polysorbate 20 (BLOTTO buffer). The blocked membrane was washed in PBS, pH 7.4, containing 0.5% polysorbate 20 for 1 h with several changes of the wash solution. Blots were incubated for 2 h at room temperature with polyclonal rabbit antibodies diluted in BLOTTO buffer to 2 μ g/ml or with human sera diluted 1:100 for detection of protease-specific IgE or 1:1,000 for detection of protease-specific IgG. After being washed, immunoblots were incubated with secondary antibodies (see above) appropriately diluted in BLOTTO buffer. After extensive washing, the blots were developed with insoluble alkaline phosphatase BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium-nitroblue tetrazolium) substrate from Boehringer.

ELISA. Binding of antiprotease antibodies was analyzed by a direct solid-phase ELISA (27). Briefly, Maxisorp polystyrene microtiter plates (Nunc, Roskilde, Denmark) were coated for 2 h at 37°C with 10 μ g of recombinant protease per ml in PBS, pH 8. The wells were then washed three times with PBS, pH 7.4, containing 0.05% polysorbate 20. Remaining free sites were blocked with PBS, pH 7.4, containing 2% (wt/vol) nonfat dry milk powder (blocking buffer) at 37°C for 1 h. After being washed, plates were incubated with sera diluted in blocking buffer containing 5% polysorbate 20 (dilution buffer) at 37°C for 2 h as follows. Serial twofold dilutions were from 1:100 to 1:12,800 for the detection of protease-specific IgG and from 1:5 to 1:640 for the detection of protease-specific IgE. Concentrations ranging from 32 to 0.015 μ g/ml were used for digoxigenin-labeled antiprotease rabbit antibodies. After a washing step, plates were incubated with the appropriate secondary antibodies. Washed plates were developed with 1 M diethanolamine buffer, pH 9.8, containing 0.5 mM MgCl₂ and 4-nitrophenyl-phosphate (1.5 mg/ml) as substrate. The A_{405} was measured with a reader from Molecular Devices (Menlo

Park, Calif.). Pooled sera from two patients with ABPA were used as a reference standard with an arbitrarily assigned 100 ELISA units per ml. The linearly titratable region of sera from patients with ABPA and healthy control individuals was expressed in a log-log manner (serum dilutions versus optical densities) and converted into ELISA units per milliliter (5). Statistical analysis was performed with the two-tailed Mann-Whitney U test. Differences associated with probability values of $P < 0.05$ were considered significant.

Competition ELISA. In a separate untreated 96-well plate (Petra Plastic, Chur, Switzerland), antigens were preincubated in dilution buffer at 37°C for 2 h with 2 μ g of digoxigenin-labeled antiprotease rabbit antibodies per ml or with serum from a patient with ABPA diluted 1:200, as appropriate. Antigens were serially twofold diluted from 100 to 0.05 μ g/ml for native protease and recombinant protease. Thereafter, the sera preincubated with antigens were transferred to washed and blocked plates coated with recombinant protease. Passive adsorption of recombinant protease to microtiter plates (Nunc), blocking, antibody incubation steps, and development were performed as described above.

RESULTS

A. fumigatus secretes an inducible alkaline protease (EC 3.4.1.14) with an apparent molecular mass of 32 kDa on SDS-PAGE (25). We recently nucleotide sequenced a genomic and a cDNA clone encoding the protease (13). The predicted sequence of the protease consists of 282 amino acid residues and is preceded by a 121-amino-acid prepro-leader peptide. Further analysis revealed that the protease does not contain cysteine residues and was not glycosylated despite the presence of four potential N-linked glycosylation sequences (Asn-X-Ser). To investigate whether IgG and IgE antibodies are elicited in response to the alkaline protease in patients with ABPA, we expressed the protease in *E. coli* as a fusion protein. The fusion tag, consisting of six adjacent histidine residues, allows purification of the recombinant protease to homogeneity by a single affinity chromatography step (10, 27, 41).

Construction of the high-level expression vector pAlp. The gene fragment encoding the mature form of the protease was amplified by PCR with a specific C primer and N primer (Table 1). As the DNA template, we used plasmid pMTL21 harboring the cDNA clone encoding the protease (13). The 862-bp PCR product was double digested with *Bam*HI-*Spe*I and subcloned into cleaved and dephosphorylated plasmid pDS56. The new vector, pAlp (Fig. 1), was used to transform *E. coli* M15 cells. Single colonies were grown in LB medium and used to prepare plasmid DNA verifying the construct pAlp (Fig. 1) by restriction analysis.

Expression and purification of recombinant protease. *E. coli* M15 cells containing the expression vector pAlp were grown in 1 liter of 2 $\times$ YT medium and induced at the mid-logarithmic point. Recombinant protease was extracted from inclusion bodies under denaturing conditions and purified by affinity chromatography with immobilized Ni²⁺-metal ion adsorbed to nitrilotriacetic acid affinity resin (10, 41). *E. coli* proteins nonspecifically adsorbed to the resin were eluted by a stepwise pH gradient from 8.0 to 6 (Fig. 2). Protease-containing fractions were eluted with 8 M urea, pH 5.5 (Fig. 2, peak D). SDS-12% PAGE gel analysis of the purified recombinant protease revealed a single band (Fig. 3) with a molecular mass of approximately 33 kDa, confirming the predicted size of the fusion protein from a nucleotide sequence of 30.4 kDa. Yields of 40 to 50 mg of virtually pure recombinant protease per liter

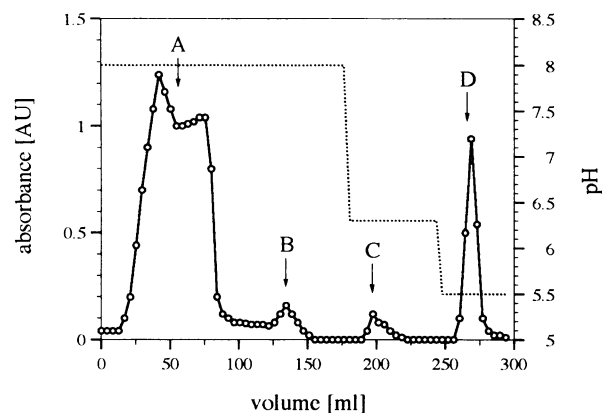


FIG. 2. Affinity chromatography elution profile of an *E. coli* lysate containing the recombinant protease expressed at high level. Bacterial lysate from 1 liter of culture was loaded onto a column at a flow rate of 0.7 ml/min. The absorbance was monitored at 280 nm (solid line) and expressed in absorbance units (AU). The pH step gradient is shown as a dotted line. The peaks are marked as follows: arrow A, sample loading; arrow B, 8 M urea (pH 8.0) wash; arrow C, 8 M urea (pH 6.3) wash; arrow D, elution peak of the recombinant protease with 8 M urea (pH 5.5).

of *E. coli* culture were obtained, as visualized by SDS-PAGE (Fig. 3).

Refolding and enzymatic activity. The recombinant alkaline protease was renatured to gain biological activity. Refolding was accomplished by extensive dilution in distilled H_2O . The recombinant protease was concentrated by ultrafiltration to an end concentration of 1 to 2 mg/ml. Assessing the proteolytic activity of the recombinant protease showed a 10-fold decrease in enzymatic activity compared with the native protease. Furthermore, the refolded recombinant protease did not show autodegradation products, as analyzed by SDS-PAGE, and the enzymatic activity could be inhibited by phenylmethylsulfonyl fluoride as described by Monod et al. (25).

Immunochemical analysis to confirm the native-like secondary structure of the recombinant protease. Correct three-dimensional structure is required for enzymatic activity as well as for immunochemical comparison of protein-antibody inter-

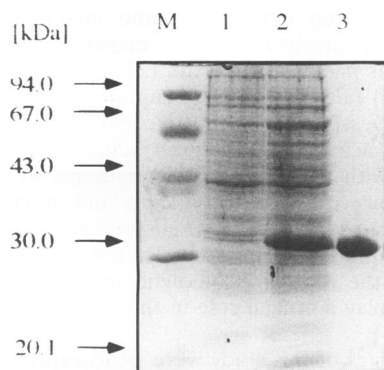


FIG. 3. Coomassie blue-stained SDS-12% PAGE gel to assay purification of the recombinant protease. Tracks: M, molecular mass markers; 1, *E. coli* cell lysate; 2, IPTG (isopropyl- β -D-thiogalactopyranoside)-induced *E. coli* cell lysate producing the recombinant protease; 3, recombinant alkaline protease (2 μ g) purified to homogeneity by affinity chromatography.

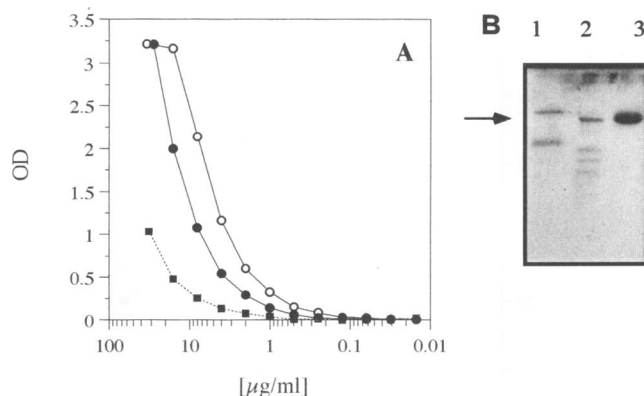


FIG. 4. Binding of rabbit sera raised against the native protease from *A. fumigatus* to the recombinant protease. (A) For ELISA, native protease ($\circ$), recombinant protease ($\bullet$), and protease from *A. oryzae* ($\blacksquare$) were coated to solid phase and showed specific binding of rabbit antisera. (B) For immunoblotting, protease from *A. oryzae* (track 1), protease from *A. fumigatus* (track 2), and recombinant protease (track 3) were separated on an SDS-15% PAGE gel (2 μ g of protein per track) and transferred to a nitrocellulose membrane. The rabbit antisera showed strong binding to recombinant and native proteases (arrow) at a molecular mass of approximately 32 kDa.

actions. In ELISA, digoxigenin-labeled antiprotease antibodies from rabbits reacted strongly to the recombinant protease as well as to the native protease (Fig. 4A). In immunoblotting assays, the native protease preparation from *A. fumigatus* appeared as a 32-kDa band (Fig. 4B) accompanied by poorly visible bands at 24 and 29 kDa, representing degradation products as stated by Monod et al. (25). The slightly larger recombinant protease was detected as a 33-kDa band because of the fusion tail and did not show autoproteolytic activity.

In order (i) to prove specificity of antiprotease rabbit antibodies and (ii) to exclude artifacts due to denaturing effects of proteins passively adsorbed to hydrophobic surfaces, we performed a competition ELISA. The binding of antiprotease rabbit antibodies to solid-phase bound recombinant protease was inhibited by increasing amounts of native and/or refolded protease in fluid phase. With both proteins, we were able to completely inhibit binding to the solid-phase-coated recombinant protease, such that 50% inhibition was reached at 1.3 μ g/ml for the native protease (Fig. 5) and at 26 μ g/ml for the recombinant protease (data not shown). Furthermore, binding of IgG antibodies from a patient with ABPA was inhibited by both native and recombinant proteases, such that 50% inhibition was achieved at 3 μ g/ml in the case of the native protease (Fig. 5) and at 75 μ g/ml for the recombinant protease.

Enzymatic activity and immunochemical analysis as well as competition ELISA results implied that the refolded recombinant protease had a conformation similar to that of the native alkaline protease from *A. fumigatus*.

Reactivity with human sera. The purity and comparable conformational structure of the recombinant protease rendered it possible to investigate sera from 19 patients with ABPA and 6 healthy control individuals to evaluate protease-specific serum IgE and IgG antibody levels.

In ELISA, protease-specific IgG antibodies from human sera reacted to solid-phase bound recombinant protease as expected. However, no significant differences between protease-specific serum IgG from patients with ABPA and healthy control individuals were observed (Fig. 6). Interestingly, none of the patients with ABPA known to have elevated levels of

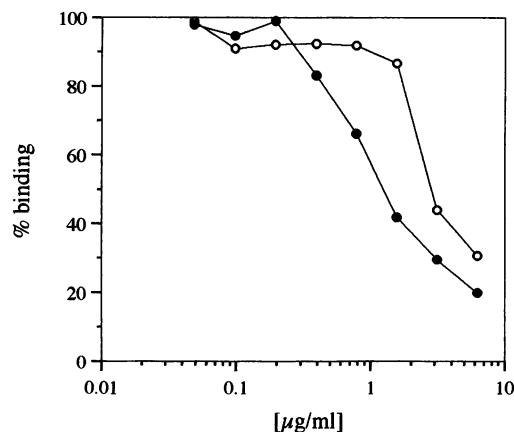


FIG. 5. Inhibition of antibody binding to solid-phase-coated recombinant protease. Rabbit antisera (●) and serum from a patient with ABPA (○) were preincubated with different concentrations of native protease. Preincubated serum samples were then transferred to recombinant-protease-coated wells, and bound antibodies were analyzed by ELISA. The absorbance value of sera preincubated without native protease was set as 100% binding.

serum IgE to *A. fumigatus* antigens expressed protease-specific IgE antibodies. Immunoblotting assays revealed that sera from patients with ABPA as well as sera from healthy control individuals showed IgG reactivity to both recombinant and native proteases (Fig. 7). The various additional IgG-binding proteins present in the native protease preparation from *A. fumigatus* represent copurified contaminants of the biochemical preparation.

Therefore, ELISA and confirming immunoblotting experiments showed strikingly that the extracellular alkaline protease did not elicit IgE antibodies in patients sensitized to *A. fumigatus*.

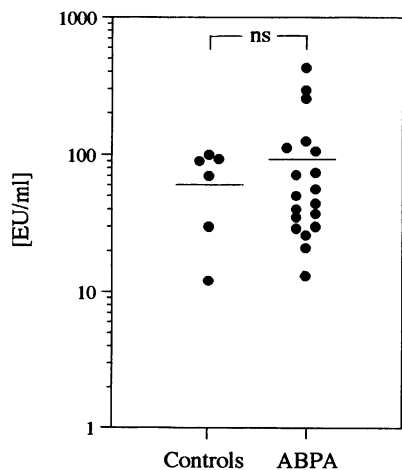


FIG. 6. Protease-specific IgG levels in sera from patients with ABPA and from healthy control individuals. The levels of serum IgG antibodies (in ELISA units [EU] per milliliter) did not show significant differences between the healthy control individuals and the patients with ABPA. Mean values are represented as lines.

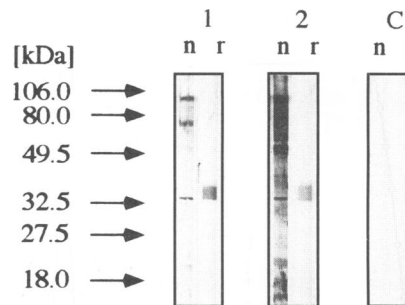


FIG. 7. IgG immunoblot of recombinant and native proteases. Quantities (2 µg) of recombinant (tracks r) or native (tracks n) protease were separated on an SDS-12% PAGE gel and transferred to a nitrocellulose membrane. Serum IgG from two patients with ABPA (tracks 1 and 2) were analyzed by immunoblotting assay and showed a band at 32 kDa corresponding to the protease. In a control experiment, the secondary antibodies used (track C) did not show binding to proteases.

DISCUSSION

Recently, we have described the cloning, expression, and purification of the recombinant *A. fumigatus* allergen I/a (27). Furthermore, we have shown that biochemically purified and apparently homogeneous *A. fumigatus* allergens may be contaminated by degradation products or by additional immunogenic and allergenic components. Since our clinical study revealed that the pure recombinant allergen I/a, in contrast to native *A. fumigatus* allergen preparations, remarkably improved the diagnosis of *A. fumigatus* allergies (26), we are interested in finding additional recombinant *A. fumigatus* allergens.

In house dust mite allergy, *Dermatophagoides* fecal extracts were reported to contain allergenic proteases (1, 40). Although the proteases secreted by *A. fumigatus* have been reported to act as allergens (32), up to now the immunological characteristics of fungal proteases in sensitized patients were not investigated. Additionally, proteases have been postulated to be important virulence factors in invasive diseases (20) and to play a key role in invasive aspergillosis (31). Strains lacking protease activity displayed reduced mortality compared with wild-type *A. fumigatus* strains when nasally introduced into the lungs of immunosuppressed mice (15). It is suggested that the invasive mechanisms of *A. fumigatus* are mediated by proteases responsible for cell detachment and airway damage (32). Evidence for an involvement of an elastolytic serine protease in aspergillosis has been reported (21, 25, 30), whereas Denning et al. (7) failed to show disruption of the elastic layers within blood vessel walls in invasive aspergillosis. The role of proteases in acting as virulence factors has further been investigated with protease-deficient mutants of *A. fumigatus* (24, 42). However, both producing and nonproducing *A. fumigatus* strains invaded lung tissue, causing comparable mortality in immunosuppressed mice (24, 43). These findings indicate that the residual proteolytic activity from other proteases could play a crucial role in the invasion of tissue by *A. fumigatus* (24).

The objectives of this study were (i) to express the alkaline serine protease from *A. fumigatus* reported by Monod et al. (13, 25) in *E. coli*, (ii) to purify the recombinant protease to homogeneity, (iii) to demonstrate the native-like structure of the recombinant protease, and (iv) to analyze the presence of protease-specific IgG and IgE antibodies in sera of individuals with or without sensitization to *A. fumigatus*.

The use of a prokaryotic heterologous expression system enabled production of large amounts of recombinant alkaline protease. The six-histidine fusion tag located at the carboxy terminus reduced the purification to homogeneity to a single affinity chromatography step. The refolded recombinant protease was then assessed for its native-like three-dimensional structure by comparing proteolytic activity and antigenic properties with those of the native protein.

The refolded recombinant protease had a low level of proteolytic activity compared with that of the native protease, a finding most probably caused by refolding problems related to leader sequences present in various proteases (12, 38). The pro-region, which is transiently covalently attached to the protease domain, promotes or facilitates correct folding (11). However, Silen and Agard (37) showed that a bacterial protease expressed without the pro-region can be folded in vitro to a native-like secondary structure but with low proteolytic activity. Additionally, our results confirmed the observation that heterologously expressed mature protease refolded in vitro failed to reach a high proteolytic activity (37).

In a protease-specific ELISA, antibodies from rabbits immunized against the native protease from *A. fumigatus* reacted strongly to solid-phase bound recombinant protease. In competition ELISA, either antiprotease rabbit antibodies or sera from patients with ABPA were preincubated with native protease in fluid phase. This resulted in a concentration-dependent decrease of binding to solid-phase-coated recombinant protease. Inhibition of binding can only be achieved if the antigen to be investigated has a native-like conformation and does not undergo extensive denaturation during the process of adsorption to the plastic surface (5).

Immunochemical comparison and proteolytic activity implied that the recombinant protease has a native-like secondary structure. Therefore, the recombinant protease was used to analyze the antigenic and allergenic characteristics in patients with ABPA, who are known to have elevated levels of *A. fumigatus*-specific IgE and IgG antibodies (9, 22). Serologic analyses of sera from 19 patients with ABPA did not show significantly elevated protease-specific IgG levels compared with those in sera from healthy control individuals. Further, we did not detect protease-specific IgE antibodies in sera from patients with ABPA. These observations suggest that the secreted alkaline serine protease from *A. fumigatus* is not an allergenic protein in patients with ABPA. The detection of IgG antibodies against the protease from *A. fumigatus* in sera from healthy control individuals was not surprising, since *A. fumigatus* is ubiquitously present in our environment. Analogous results were already reported from analysis of sera from healthy control individuals using the recombinant *A. fumigatus* allergen I/a (26, 27).

Although the secreted 32-kDa alkaline protease of *A. fumigatus* does not elicit an IgE antibody response in patients with ABPA, it undoubtedly plays a role in tissue invasion by *A. fumigatus*. Correlation between protease activity and the ability to cause invasive aspergillosis had been shown by Kothary et al. (16) in a murine model, whereas Reichard et al. (30) reported detection of a serine protease in mycotic human lung tissue. Furthermore, it was suggested that the potential of *A. fumigatus* proteases to damage the airway epithelium in patients with ABPA might play a role in triggering the allergic response (32). The resulting cell damage could lead to increased penetration of *A. fumigatus* antigens and allergens into the submucosal tissue and hence exacerbate an existing allergic response of patients sensitized to *A. fumigatus*. Since the allergy-promoting potential of proteases might be important in *A. fumigatus*-associated respiratory diseases or invasive as-

pergilloma, further investigations will be carried out to elucidate the protease-dependent pathophysiological mechanisms.

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