Molecular and Cellular Biochemistry **252:** 157–163, 2003.

Expression and characterization of Asp fI, an immunodominant allergen/antigen of *A. fumigatus* in insect cell

Priyanka Priyadarsiny,[1] Prabodha K. Swain[2] and P. Usha Sarma[1]

[1]*Institute of Genomics and Integrative Biology, Mall Road, Delhi, India;* [2]*W.K. Kellogg Eye Centre, University of Michigan, Ann Arbor, MI, USA*

Received 18 June 2002; accepted 10 February 2003

Abstract

Asp fI is a major allergen/antigen/cytotoxin of *Aspergillus fumigatus* and exhibits ribonuclease activity. This allergen plays a role in allergic and invasive Aspergillosis and reported as a major cytotoxin with ribonuclease activity. To express the protein in large quantity and to characterize the multifunctional nature of Asp fI, we have generated recombinant baculovirus by introducing the gene in pFastBac HTa expression vector and expressed in insect cell. The baculovirus expression vector system has been used as a versatile system for the efficient expression of proteins with most eukaryotic posttranslational modification. Recombinant Asp fI was expressed as ~1% of the total cellular protein in infected Sf9 insect cells. The protein was purified using Ni^{2+} affinity column chromatography and the yield of purified protein was ~10 mg/1 g of total cellular protein. Immunoreactivity of the protein was determined by immunoblot analysis using both poly His monoclonal antibody, IgG and IgE antibodies present in the sera of ABPA patients. The protein was glycosylated as revealed by the glycoprotein staining and was observed to retain both ribonuclease and cytotoxic activities. These results suggest that Asp fI expressed in insect cell was post translationally modified and biologically active that can be used as a diagnostic marker for biochemical studies. (Mol Cell Biochem **252**: 157–163, 2003)

Key words: *Aspergillus fumigatus*, ribotoxin, eukaryotic expression

Introduction

Asp fI is a multifunctional protein secreted by *A. fumigatus* and implicated in pathogenesis of Aspergillus related disorders. It is a Concanavalin A (ConA) non-binding allergen inducing type I and III hypersensitivity reactions in allergic form of aspergillosis [1]. Asp fI was reported as a ribotoxin and it has structural and functional similarities with α-sarcin, mitogillin and restrictocin, which are ribotoxin secreted by the nonpathogenic strains of Aspergillus species [2–6]. These toxins hydrolyze the 28S rRNA and inhibit protein synthesis leading to cytotoxicity in eukaryotic cells [1–4]. Few cytotoxic ribonucleases are glycoproteins and their glycan moieties are important for internalization or transmembrane localization of the toxin [7].

However, recent observation on binding of Asp fI towards lung surfactant proteins SP-A and SP-D which has structural homology with C1q lectin suggests presence of carbohydrate moieties on the surface of the protein. Several groups have studied the interaction of lung surfactant protein SP-A and SP-D with glycosylated antigen/allergens of *A. fumigatus*, which inhibit specific IgE binding and block the histamine release from sensitized basophils [8, 9]. In another study it has been shown SP-A and SP-D bind to the glycosyl moieties of the allergens (45 and 55 kDa) of *A. fumigatus* and inhibit specific IgE binding to the allergens [9, 10]. Inhibition of IgE binding activity of Asp fI was also observed in presence of lung surfactant protein suggests the involvement of carbohydrate moiety in immunological activities of the protein (thesis submitted 1999 to University of Delhi by Priyadarsiny *et al.*).

Present address: P.K. Swain, National Brain Research Center, Gurgaon, Haryana-122001, India
Address for offprints: P. Usha Sarma, Institute of Genomics and Integrative Biology, Mall Road, Delhi-7, India (E-mail: u_sarma@hotmail.com)

Moreover a number of immunodominant glycoprotein allergens and antigens of *A. fumigatus* are glycoproteins with catalytic activities. [9–11]. The biochemical functions of these proteins may contribute to the pathogenesis of the disease. The recombinant Asp fI expressed in prokaryotic systems didn't elaborate on the biological activity such as ribonuclease activity and cytotoxicity. Expressed recombinant Asp fI showed 50% reduced ribonuclease activity and specific cytotoxic activity was absent compare to the native protein which could be due to the lack of post translational modification in the protein expressed in *E. coli* [13].

To address these problems, in the current report we expressed Asp fI protein in insect cells using recombinant baculovirus expression system. Expressed recombinant Asp fI was purified by single step purification method using Ni^{2+} affinity chromatography. Protein was characterized for the post translational modification and immunological activity. Purified protein showed specific binding to IgG and IgE antibody present in the sera of ABPA patients. The protein retained its ribonuclease activity and was toxic to the mouse macrophages similar to that of native Asp fI. High level expression of biologically active rAsp fI in Sf9 insect cells suggest that in this system Asp fI protein can be generated as a heterologous source which can be used in diagnostics and for structure function studies.

Materials and methods

Genomic DNA purification, PCR amplification and cloning of Asp fI gene

Briefly, the genomic DNA was isolated from the mycelial extract of 4-day-old *A. fumigatus cultures* (strain no. 2605 from the patients sputum cultured at V. P. Chest institute, Delhi [14]. Asp fI gene was PCR amplified from the genomic DNA of *A. fumigatus* and initially cloned in TA-cloning vector (Clontech). DNA isolated from the positive clone was further PCR amplified with PP1[GCA AAG CTT GCG ACC TGG ACA TGT ATA AAC CAG CAG] and PP2 primers [CGG GAT CCC TAA TGA GAA CAC AGT CTC AA] to incorporate *Bam HI* restriction site after stop codon. Amplified DNA was treated with Klenow polymerase to make blunt ends and purified using phenol-chloroform extraction followed by ethanol precipitation. Purified DNA was digested with *EcoRI* and the gel extracted to subclone in pFastbac HTa vector (BRL Life Technology) digested with *Eco*RI – *Stu* I and recombinant baculovirus construct is designated as pFastBac HTa-AspfI. The Asp fI gene was sequenced using PP2 and M13 forward primers in a dideoxy chain termination Cycle Sequencing kit (Amersham) and proper reading frame was verified before use in expression studies. Asp fI bacmid was generated by homologous recombination of pFastBac HTa-Asp fI plasmid with the bacmid DNA present in DH10 Bac competent cells. Transformation and screening of positive clones were performed according the protocol suggested by the manufacturer (BRL Life Technology).

Expression and purification of recombinant Asp fI

Sf9 insect cells were maintained in SFM (ATCC) containing 5 µg/ml streptomycin and 5 U/ml penicillin at 27°C. Cells in a six-well plate was transfected by using CELL FECTIN reagents (Life Technologies) at 80% cell confluency. In brief: 5–10 µg of bacmid DNA diluted in 100 ul of Opti-MEM was mixed with 20 µl of CELL FECTIN diluted in equal volume of Opti-MEM. The mixture was incubated at room temperature for 45 min. The transfection cocktail was diluted further upto 1 ml in Opti-MEM before layered on Sf9 cells and incubated for 5 h at 27°C. After the incubation transfection cocktail was substituted with the SFM till it was harvested after 72 h post transfection and characterized for the expression of recombinant protein.

Sf9 insect cells were transfected at 0.1 multiplicity of infection (MOI) for the expression of the recombinant Asp fI protein. After 72 h post infection cells were harvested and resuspended in PBS. The cells were centrifuged at 500 × g for 5 min to get rid of serum PBS and pellet was resuspended in lysis buffer (50 mM Tris-HCl pH 8.5, 10 mM 2 mercaptoethanol, 1 mM PMSF, 1% NP-40 at 4°C). Insoluble debris was removed by centrifugation at 10,000 × for 10 min and the cell supernatant was diluted in Buffer A (20 mM Tris HCl, pH 8.5, 500 mM KCl, 10 mM 2-mercaptoethanol, 10% glycerol) to incubate with equilibrated Ni^{2+} resin. Protein bound resins were packed in a column at 4°C and washed extensively using 5–10 vol. of wash buffer (20 mM Tris pH 8.0, 500 mM KCl, 20 mM imidazole, 10 mM 2 mercaptoethanol, 10% glycerol) in a flow rate of 0.5 ml/min. The column was re-equilibrated with 10 vol. buffer A and the bound protein was eluted with 5–10 vol. of Buffer B (20 mM Tris HCl pH 8.5, 100 mM KCl, 0–50 mM imidazole, 10 mM 2-mercaptoethanol, 10% glycerol. Eluted fractions (0.5 ml) were collected in separate tubes and analyzed the protein by SDS PAGE.

SDS-PAGE and immunoblot

Proteins were separated on 15% denaturing gel by SDS-PAGE [15] and transferred to the nitrocellulose membrane (Schleicher & Schell, 0.45 µ pore size) as described by Towbin [16]. The membrane was blocked for 1 h in PBS containing 3% nonfat milk at room temperature and incubated with poly-His (1:1000 dilution) or anti-Asp fI (1:100 dilution) monoclonal antibody for 3 h at 37°C. Membrane was washed in PBS-0.1% Triton X-100 solution and incubated for 2 h with anti

mouse IgG peroxidase (1:2000 dilution in PBS). Finally the membrane was washed in PBS–0.1% Triton X-100 solution and the immunoblot was developed using DAB (diamino benzidine)-H_2O_2 solution.

Carbohydrate staining

Purified recombinant Asp fI protein was analysed on the SDS-PAGE and stained for glycoprotein using the silver staining method [17]. The sample was electrophoresed on SDS-PAGE gel and fixed overnight in 200 ml of solution containing 25% isopropanol and 7% acetic acid. The gel was incubated with oxidizing solution (1.05 g of periodic acid in 150 ml of distilled water + 4 ml of 25% isopropanol in 7% acetic acid) for 5 min followed by extensive washing with distilled water. The gel was incubated for 10 min with silver nitrate staining solution (28 ml of 0.1 N NaOH, 1 ml of ammonium hydroxide, 5 ml of 20% silver nitrate and 115 ml of distilled water) and developed with the developing solution (50 mg of citric acid, 0.5 ml of formaldehyde (37%) in 1 litre of distilled water). Finally 0.35% acetic acid was added to the gel for neutralization.

Toxicity assay

Murine macrophage cells (Raw264.7, J774) were maintained in DMEM containing 10% FCS, 100 µg/ml streptomycin and 100 U penicillin at 37°C with 5% CO_2. Equal numbers of cells were cultured in a 96-well plate for cytotoxicity assay. Varying concentrations of Asp fI protein, purified from *A. fumigatus* as well as purified rAsp fI expressed in insect cells were diluted in DMEM and added in triplicate to the cultured cells [18]. Cytolysis was monitored after 8 h by MTT assay. In brief: 1.5 mg/ml MTT was added to the culture media and the cells were incubated for 30 min. Then the culture media was substituted with 100 µl of acidified isopropanol solution per well to lyse the cells. The absorbance of the supernatant was measured at 570 nm and analyzed as percent cell viable to that of the control (without any protein treatment) [18].

Ribonuclease activity

Different concentrations of Asp fI (0.1–1µg) were incubated with 1% yeast RNA in 0.1M acetate buffer, pH 5.0 for 3 h at 37°C. After 3 h, RNA was precipitated with 0.5 ml of 75% uranyl acetate in 25% of perchloric acid at 4°C. The reaction mixture was centrifuged at 13,000 rpm for 10 min and supernatant containing soluble nucleotides was measured at A_{260}. One unit of ribonuclease is defined as the amount of protein required to produce a δA_{260} of 1.0 of acid soluble nucleotides when incubated with 1% substrate RNA for 15 min at pH 7.4 in a reaction volume of 1.5 ml.

Results and discussion

Cloning expression and purification of Asp fI gene in insect Sf9 cells

Asp fI gene spans 450 bp in the fungal genome and the transcript encodes a protein of ~18 kDa consisting 149 amino acids. PCR amplified Asp fI gene was cloned down stream of polyhedrin promoter in *Eco*RI and *Stu*I sites of pFastBac HTa transfer plasmid. Recombinant bacmid was generated by site-specific recombination between transfer plasmid pFastBac HTa containing Asp fI and bacmid in host *E. coli* (DH10 BAC). Sf 9 cells infected with recombinant baculovirus was analyzed for expression of Asp fI. Analysis of the insect cell proteins expressing Asp fI comprise ~1% of the total cellular protein determined by densitometric analysis of protein band.

For large-scale purification, Sf9 cells were infected with the recombinant baculovirus at 5 MOI. Insect cells were lysed to release the expressed protein in the supernatant. Recombinant protein with poly-HIS tag was bound to Ni^{2+} affinity resins and eluted with the buffer containing 20 mM Imidazole. Our previous attempt to express Asp fI in *E. coli* resulted insoluble protein, localized in the inclusion bodies. Lack of post-translational modification necessary for proper folding and solubility of the Asp fI protein could be responsible for producing insoluble protein in *E. coli*. Since prokaryotic expression hosts lack the machinery for post translational modification such as glycosylation, phosphorylation necessary for proper folding and solubility of the eukaryotic proteins, insect cell (baculovirus expression system) was used to study the expression of Asp fI. Recombinant rAsp fI was expressed in Sf9 insect cells and soluble protein purified by affinity chromatography was analysed by 15% SDS-PAGE. Coomassie blue stained gel showed a major 18 kDa homogenous protein band in the lane containing whole insect cell lysate infected with baculovirus (Fig. 1). Molecular weight of rAsp fI (18 kDa) was similar to native Asp fI (purified from synthetic culture of *A. fumigatus*) but higher than the protein expressed in *E. coli* [5, 13]. Such discrepancy in the molecular weight of the rAsp fI could be the result of post translational modification in the protein.

Immunological activity of rAsp fI

rAsp fI immunologically specific to IgG and IgE present in the ABPA patients

The specificity of the purified Asp fI was analysed in the Western blot analysis using poly-His monoclonal antibody. A protein band of 18 kDa was detected in the Western blot probed with poly-HIS monoclonal antibody (Fig. 2). Mono-

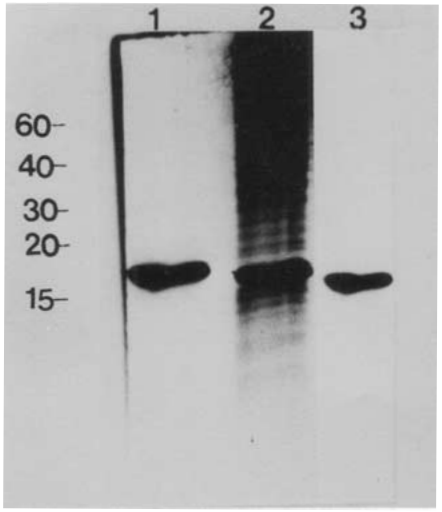

Fig. 1. SDS-PAGE analysis of rAsp fI expressed in Sf9 insect cell transfected with recombinant bacmid. Lane 1 – purified rAsp fI; lane 2 – expressed rAsp fI in cell lysate of Sf 9 cell; lane 3 – native Asp fI.

clonal antibody used was specific to the poly-His (hexa-histidine) tag confirming the expressed protein is a fusion protein with histidine tag and matched with the expected molecular weight (18 kDa) as has been observed with native Asp fI protein.

Asp fI is a major antigen/allergen that binds specifically to the IgG and IgE antibodies present in the serum of ABPA patients [1, 3]. Several groups have also identified the epitopic region of Asp fI which demonstrated distinct IgE antibody binding response against sera from ABPA patients and proliferative responses in peripheral blood mononuclear cells from the patients [19, 20].

To determine if the baculovirus expressed rAsp fI retains the identical immunoreactivity with the IgG and IgE antibody of the ABPA patients, immunoblot of rAsp fI was treated with ABPA patient's sera. Baculovirus expressed rAsp fI exhibited specific binding to both IgG and IgE antibodies present in the patient's serum similar to native Asp fI (Fig. 3). The results suggest that the protein retains its antigenicity and allergenicity comparable to the native fungal Asp fI protein. The purified recombinant antigen rAsp fI can be used for screening the serum of ABPA patients and for immunodiagnosis of aspergillosis.

rAsp fI, a Glycoprotein

To characterize the post-translational protein modification of rAsp fI, the purified protein was subjected to glycoprotein staining [17]. The rAsp fI migrated as an 18 kDa protein band stained positive in glycoprotein staining (Fig. 4). This finding substantiates our earlier observation that native Asp fI interacts with different lectins such as GNA, PNA, MAA, SNA and DSA (data not shown). Baculovirus expression system is

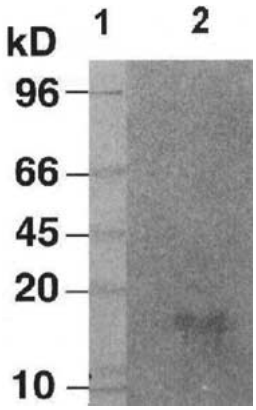

Fig. 2. Western blot analysis of rAsp fI against poly-histidine monoclonal antibody. Lane 1 – protein molecular weight markers; lane 2 – purified rAsp fI expressed in Sf9 cell.

known to produce necessary glycosylation in the protein. It is worth mentioning that sometimes hyper-glycosylation occurs through baculovirus expression system that helps to identify potential glycosylation domains in the protein. In case of rAsp fI hyperglycosylation could not be the case since the expressed protein has a molecular weight identical to the native protein expressed in *A. fumigatus*.

Some of the glycoproteins ranging from 20–90 kDa are characterized as allergens secreted by *A. fumigatus* [21–23]. Carbohydrate moieties of many fungal and plant glycoproteins have been shown to be responsible for specific IgE binding activity. Recent report on Asp f2 protein of *A. fumigatus* showed specific binding of Asp f2 to IgE antibodies in the sera of ABPA patients. Comparison of amino acid sequence of Asp f2 showed homology with a 37 kDa ConA nonbinding protein with possible glycosylation sites [11, 12]. The

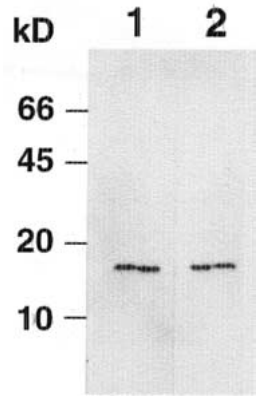

Figure 3. Immunoblot analysis of rAsp fI using pooled serum of ABPA patients. Membrane strips immobilized with purified rAsp fI were detected for specific immunoglobulin binding by Western blot analysis. Lane 1, IgG antibody; lane 2, IgE antibody.

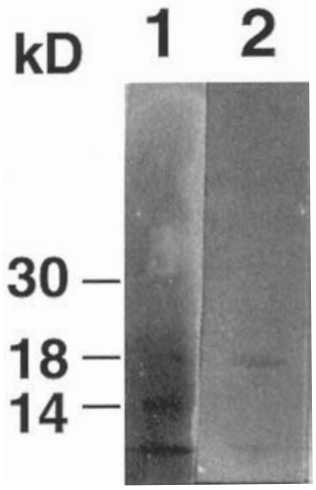

Fig. 4. Carbohydrate staining of rAsp fI. Lane 1 – protein molecular weight marker; lane 2 – Purified rAsp fI protein.

allergenic proteins, gp45 and gp55 of *A. fumigatus* have been characterized as N-linked glycoproteins and deglycosylation abolishes their binding to IgE in the ABPA patient's serum [21–23]. The IgE binding of these allergens was inhibited in presence of SP-A and SP-D, which bind through their carbohydrate recognition domains suggesting that SP-A and SP-D may be involved in the modulation of allergic sensitization [8, 9]. The IgE binding activity of Asp fI was also significantly inhibited in presence of lung surfactant protein .The role of carbohydrate moieties in the immunological activities of the protein needs to be investigated.

Ribonuclease activity of rAsp fI

Asp fI exhibited ribonuclease activity in *in vitro* experiments [2, 4]. Similar studies were carried out to determine the ribonuclease activity of rAsp fI purified from the insect cell. The degradation pattern of the yeast RNA incubated with native and rAsp fI are identical (data not shown). The specific activity of rAsp fI was determined as 102 kunitz/mg in comparison to 108 kunitz/mg in case of native Asp fI (Table 1). The purified rAsp fI produced enhanced ribonuclease activity than the crude insect cell lysate containing rAsp fI. This observation suggests that the activity is specific to the purified protein. Control buffers do not produce any activity (data not shown). The ribonuclease activity observed in case of the rAsp fI was comparable to that of native Asp fI and bovine RNase A protein. Earlier reports on recombinant Asp fI protein in *E. coli* showed only 50% ribonuclease activity compared to the activity of native protein [13]. These results suggest a possible role of carbohydrate moiety in the ribonuclease activity of rAsp fI.

Table 1. Ribonuclease activity of native and purified recombinant Asp fI protein

Units of ribonuclease activity	(kunitz/mg)
Native 18 kDa	108
Purified rAsp fI	102
rAsp fI in Sf9 cell lysate	86
Ribonuclease A	96

Glycosylation appears to contribute natural conformation and ribonuclease activity of the Asp fI protein. The functional domain of Asp fI is primarily dependent on specific amino acid residues like His, Cys, Glu, which are generally involved in the RNase mechanism [4]. Native Asp fI and α-sarcin produce a fragment of 400 bp RNA by the specific cleavage of 28S rRNA [2–4]. However, the catalytic domain in Asp fI is not yet identified.

Cytotoxicity assay of rAsp fI

Native Asp fI is toxic to mouse macrophage cells. Cytotoxic activity of purified rAsp fI was studied in an identical assay system and compared with that of native Asp fI, purified from the fungus (Fig. 5). Cytotoxic activity of rAsp fI increased in a dose dependent manner at a concentration range of 125–1000 ng/ml for both J774A.1 and RAW264.7 mouse macrophages. IC_{50} of the purified rAsp fI was found to be 250 ng while IC_{50} of the native Asp fI is 80 ng/ml. However, Asp fI expressed in insect cells did not produce any cytotoxic effect to the host insect cells. Though the mechanism of Asp fI internalization and specific substrate for ribonuclease activity in the cell is still not known, it is hypothesized that the mechanism may be similar to α–sarcin, which binds preferentially to negative charge inducing phospholipid

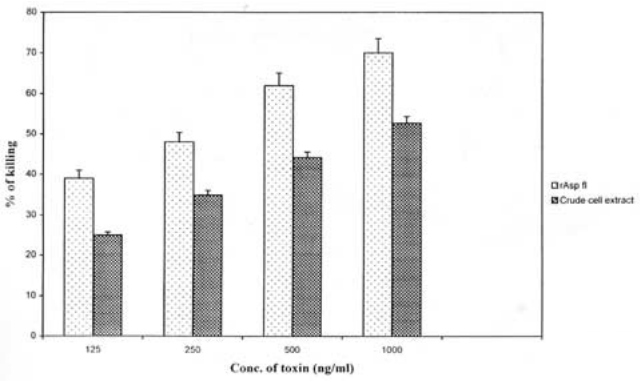

Fig. 5. Cytotoxic activity of rAsp fI on macrophages (RAW 264.7). Crude cell extract represented as heavy dots and purified rAsp fI are represented as light dots in the toxicity graph.

aggregation and fusion of lipid vesicles for the internalization [2].

Presence of ribonuclease activity of rAsp fI suggests the inhibition of protein synthesis by hydrolysing the phosphodiester bond of 28S rRNA similar to the native Asp fI. It has been reported that some of the ribotoxins enter into the cell by endocytosis [24]. After vesicular transport, the toxin crosses an intracellular membrane in order to reach its substrate. Molecular modeling studies of restrictocin suggest ability of RNase to cross the cell membrane through a loop, which is similar to lectin sugar binding domain [25]. As Asp fI has sequence homology with restrictocin, analysis of secondary structure of Asp fI could give some important clue for the catalytic sites involved in the function.

Asp fI, a multifunctional glycoprotein secreted from the pathogenic species of Aspergillus is structurally and functionally similar to ribotoxin of a non-pathogenic species of Aspergillus genus. Asp fI could play a dual role in the pathogenesis of *A. fumigatus* related disorders (a) by promoting fungal colonization through cytotoxic activity (b) inducing inflammatory reaction involving IgE antibodies present on human lung mast cell [26].

Current study was undertaken with a view to examine the role of glycosylation in the biological activities of Asp fI, which is a con A non-binding immunodominant protein. Presence of ribonuclease and cytotoxic activity of rAsp fI indicated that post translational modification might play a role in its biological function. In view of the multifunctional nature of this allergen, site directed mutagenesis can be used to identify immunodominant region of the molecule which could help in delineating the functional domain of the allergen ribonuclease and identify the pockets in the secondary structure responsible for its biological function. Current work opens up the scope of using this Aspergillus allergen as a model for understanding the structure-function relationship of the pathogen related proteins and the mechanism involved in the pathogenesis of Aspergillosis.

Acknowledgements

We thank the University Grant Commission and Council of Scientific and Industrial Research for their financial support.

References

1. Arruda LK, Platts Mills TAE, Fox JW, Chapman MD: *Aspergillus fumigatus* allergen I, a major IgE binding protein, is a member of the mitogillin family of cytotoxins. J Exp Med 172: 1529–1532, 1992
2. Endo Y, Huber PW, Wool IG: The ribonuclease activity of the cytotoxin alpha sarcin. J Biol Chem 258: 2662–2667, 1982
3. Lamy B, Moutaoukil M, Latge JP, Davies JP: Secretion of a potential virulence factor, a fungal ribonuclease during human aspergillosis infections. Mol Microbiol 7: 1811–1815, 1991
4. Madan T, Arora N, Sarma PU: Ribonuclease activity dependent cytotoxicity of Asp fI, a major allergen of *A. fumigatus*. Mol Cell Biochem 175: 21–27, 1997
5. Arruda LK, Mann BJ, Chapman MD: Selective expression of a major allergen cytotoxin Asp fI in *A. fumigatus.* J Immunol 149: 3354–3359, 1992
6. Latge JP, Moutauukil M, Debeaupis JP, Bouchaara JP, Haynes K, Prevost MC: The 18 kDa antigen secreted by *A. fumigatus.* Infect Immun 59: 2586–2594, 1991
7. Yang X, Moffat K: Insights into specificity of cleavage and mechanism of cell entry from the crystal structure of highly specific Aspergillus ribotoxin. Structure 4: 837–852
8. Madan T, Kishore U, Singh M, Strong P, Clark H, Hussain EM, Reid KB, Sarma PU: Surfactant proteins A and D protect mice against pulmonary hypersensitivity induced by *Aspergillus fumigatus* antigens and allergens. J Clin Invest 107: 467–475, 2001
9. Madan T, Kishore U, Shah A, Eggleton P, Strong P, Wang JY, Aggrawal SS, Sarma PU, Reid KB: Lung surfactant proteins A and D can inhibit specific IgE binding to the allergens of *Aspergillus fumigatus* and block allergen-induced histamine release from human basophils. Clin Exp Immunol 110: 241–249, 1997
10. Madan T, Banerjee B, Bhatnagar PK, Shah A, Sarma PU: Identification of 45 kD antigen in immune complexes of patients of allergic bronchopulmonary aspergillosis. Mol Cell Biochem 166: 111–116, 1997
11. Banerjee B, Greenberger PA, Fink JN, Kurup VP: Immunological characterization of Asp f2, a major allergen from *Aspergillus fumigatus* associated with allergic bronchopulmonary aspergillosis. Infect Immunol 66: 5175–5178, 1998
12. Banerjee B, Kurup VP, Greenberger PA, Hoffman DR, Nair DS, Fink JN: Purification of a major allergen, Asp f2 binding to IgE in allergic bronchopulmonary aspergillosis, from culture filtrate of *Aspergillus fumigatus*. J Allergy Clin Immunol 99: 821–827, 1997
13. Moser M, Carmerl R, Menz G, Schneider T, Dudler T, Virchow C, Gmachil M, Blaser K: Cloning and expression of recombinant *Aspergillus fumigatus* allergen I/a (rAsp fI) with IgE binding and type I skin test activity. J Immunol 149: 454–460, 1992
14. Bir N, Paliwal A, Muralidhar K, Reddy P, Sarma PU: Prep Biochem 25: 171–181, 1995
15. Laemmli UK: Cleavage of structural protein during assembly of the head of bacteriophage T4. Nature (Lond) 227: 680–685, 1970
16. Towbin H, Stahelin T, Gordan J: Electrophoresis transfer of proteins from acrylamide gels into nitrocellulose sheets. Proc Natl Acad Sci USA 76: 4350–4354, 1979
17. Penny J, Hitchcock, Teresa M, Brown: Morphological heterogeneity among Salmonella lipopolysaccharide chemotypes in silver stained polyacrylamide gels. J Bacteriol 269–277, 1983
18. Madan T, Arora N, Sarma PU: Identification of a major cytotoxin of *A. fumigatus*. Mol Cell Biochem 167: 89–97, 1997
19. Sarma PV, Purkayastha S, Madan T, Sarma PU: Expression of an epitopic region of Asp fI, an allergen/antigen/cytotoxin of *Aspergillus fumigatus*. Immunol Lett 70: 151–155, 1999
20. Kurup VP, Banerjee B, Murali PS, Greenberger PA, Krishnan M, Hari V, Fink JN: Immunodominant peptide epitopes of allergen, Asp f1 from the fungus *Aspergillus fumigatus*. Peptides 19: 1469–1477, 1998
21. Banerjee B, Madan T, Sharma GL, Prasad HK, Nath I, Sarma PU: Characterisation of a glycoprotein antigen (45 kDa) of *Aspergillus fumigatus*. Serodiag Immunother Infect Dis 7: 147–152, 1995
22. Fratamico PM, Buckley HR: Identification and characterisation of an immunodominant 58-Kilodalton antigen of *Aspergillus fumigatus* recognized by sera of patients with invasive aspergillosis. Infect Immun 309–315, 1991

23. Teshima R, Ikebuchi H, Sawada J, Miyachi S, Kitani S, Iwama M, Irie M, Ichinoe M, Terao T: Isolation and characterization of a major allergenic component (gp55) of *Aspergillus fumigatus*. J Allergy Clin Immunol 92: 698–706, 1993
24. Deurs BV, Petersen OW, Olsnes S, Sandvig K: The way of endocytosis. Int Rev Cytol 117–131, 1989
25. Bufe A, Schramm G, Kenow M, Schalaak M, Becker WM: Major allergen Phl pVb in timothy grass in novel pollen RNase. FEBS Lett 363: 6–12, 1995
26. Purkayastha S, Madan T, Shah A, Krishnamurthy HG, Sarma PU: Multifunctional antigens of *A. fumigatus* and specific antibodies. Appl Biochem Biotechnol 83: 271–283; 297–313, 2000