

Ribonuclease activity dependent cytotoxicity of Asp fl, a major allergen of *A. fumigatus*

Taruna Madan, Naveen Arora and P. Usha Sarma

Centre for Biochemical Technology, Delhi-110007, India

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Abstract

A major allergen/antigen, Asp fl, secreted by *Aspergillus fumigatus* exhibits cytotoxicity towards eukaryotic cell lines. Asp fl inhibited protein synthesis in RAW cells with an IC_{50} of 4.5 nM and also degraded ribosomal RNA of RAW cells at a similar concentration. Ribosomal inactivation by Asp fl may be the probable mechanism for protein synthesis inhibition. Specific ribonuclease activity of Asp fl was observed to be 100,000 U/mg. Presence of strong RNase activity in Asp fl was further confirmed by agar gels containing yeast RNA. Electrophoretic run on agarose gels showed that Asp fl degrades all species of naked RNA. Modification of histidine residues of Asp fl with diethyl pyrocarbonate and alkylation of cysteines with iodoacetamide resulted in loss of ribonuclease activity and cytotoxicity of Asp fl. The current study establishes the ribonuclease activity of a purified major allergen of *A. fumigatus* that inhibits protein synthesis and kills the eukaryotic cells. (Mol Cell Biochem 175: 21–27, 1997)

Key words: *Aspergillus fumigatus*, ribonuclease, cytotoxicity, Asp fl

Introduction

A. fumigatus antigens are complex glycoproteins, glycans and proteins with a variety of biological functions [1]. Antigens with collagenase, elastase and protease activities have been reported from different isolates of the fungi [2–4]. The biochemical functions of various antigens and immune responses to some of the antigens contribute significantly to the pathogenesis of *Aspergillus* induced infections [5]. A detailed investigation on the biochemical properties of immunoreactive antigens of *A. fumigatus* would facilitate understanding on diagnosis and pathogenesis of Aspergillosis.

Asp fl, an 18 kDa protein secreted by *A. fumigatus*, is reported to be a major allergen/antigen involved in the pathogenesis of *Aspergillus* related disorders [6, 7]. An IgE mediated response to Asp fl has been reported in 85% of the patients with *A. fumigatus* induced allergic disorders [6]. The 18 kDa protein is also a major antigen found in the urine of the patients with invasive aspergillosis [8]. The cytotoxic nature of Asp fl has been established on different eukaryotic cell lines [9].

Recombinant Asp fl was also observed to be cytotoxic to EBV transformed PBMC's of ABPA patients [10].

Cloning and sequencing of the gene for 18 kDa allergen from two different isolates in different vectors showed homology of the sequence with fungal toxins restrictocin, mitogillin and alpha-sarcin [7] (Sarma *et al.* unpublished data). These toxins are known to kill cells by inhibiting protein synthesis by hydrolysing the phosphodiester bond of 28 S rRNA [11]. Asp fl inhibited protein synthesis in cell free reticulocyte lysates [6] and produced a c.400 base fragment from the 28S rRNA [10].

Although the mechanism of cytotoxicity of Asp fl appears to be ribosome inactivation, precise mechanism of cytotoxicity is yet to be established. Current study was undertaken to evaluate the effect of Asp fl on protein synthesis in eukaryotic cells, to analyse and characterise ribonuclease activity of Asp fl and to determine the correlation of its ribonuclease activity and cytotoxic activity. Understanding on the mechanism of cytotoxicity of Asp fl will also facilitate the use of this cytotoxin in chemotherapy of cancers.

Materials and methods

Purification of Asp fl

Asp fl was purified as reported previously [9]. Three week culture filtrate (enriched protein antigenic fraction) of *A. fumigatus* isolated from an ABPA patient was subjected to phenyl Sepharose chromatography. Fractions were obtained on successive elution with a gradient of decreasing concentrations of ammonium sulfate (25, 20, 15, 10, 5, 0%) and simultaneously increasing concentrations of ethylene glycol (0, 10, 20, 30, 40, 50%). Fraction III (15% ammonium sulfate + 20% ethylene glycol) was subjected to reverse phase HPLC on C-18 column using a 10–70% gradient of acetonitrile in water containing 0.1% v/v trifluoroacetic acid.

Cell lines

RAW (macrophage) cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 2 mM L-glutamine, 25 mM HEPES buffer, 10% FCS and 10 µg/ml gentamicin. The cells were maintained at 37°C in 5% CO₂ in a humidified atmosphere.

Assay of protein synthesis

Assay of protein synthesis by RAW cells in presence of purified Asp fl was carried out as described previously [12]. Cells were scrapped, centrifuged, resuspended in fresh medium and dispensed into 96 well plates at a density of 10⁴ cells/100 µl/well. On the following day cells were incubated with various concentrations of purified Asp fl (0–500 ng/ml) for 8 h. In control wells no antigen was added. Leucine free medium containing ¹⁴C labelled leucine (1 µCi/ml, BARC, India) was added and cells were further incubated for 12 h. Medium was aspirated and cells were incubated with 10% trichloroacetic acid for 30 min at 4°C and were subsequently washed with 5% trichloroacetic acid. The cells were lysed with 1 N NaOH and the lysate was adsorbed on to the filter paper discs. Dried discs were transferred to scintillation fluid and the counts were taken in a beta-counter. Experiment was carried out twice and each sample was taken in triplicate.

Identification of ribonuclease activity

The ribonuclease activity of purified Asp fl was examined and estimated using a qualitative detection assay as described by Holden and Pirie [13]. 100 ml of 1% agarose was prepared and cooled to 45°C to which 10 ml of 2% solution of commercial yeast RNA in 0.1 M acetate buffer, pH 5 was added

and solution was poured into polystyrene petridishes to a depth of 3 mm. Protein enriched antigenic fraction, purified Asp fl, chemically inactivated Asp fl and control (buffer with no Asp fl) were spotted on the agar plates (10 µl each containing 7.5 µg of protein). The plates were incubated for 18 h at 25°C. Undigested RNA was precipitated in the gel by filling the dishes with a 0.25% w/v solution of uranyl acetate in 1.25% w/v trichloroacetic acid. Approximate activity of the ribonuclease on the gel was estimated by measuring the diameter of the spots and comparing with that of known amounts of pancreatic RNase (Sigma).

Ribonuclease assay

Specific activity of ribonuclease was calculated spectrophotometrically at 260 nm, various concentrations of pancreatic RNase and Asp fl were incubated with 1% w/v yeast RNA in 0.1 M phosphate buffer, pH 7.4 for 15 min at 37°C. RNA was precipitated with 0.5 ml of 0.75% uranyl acetate in 25% perchloric acid at 4°C. The reaction mixtures were centrifuged and supernatant containing acid soluble nucleotides were measured at 260 nm. One unit of ribonuclease is defined as the amount of protein required to give a δA_{260} 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.

Ribonuclease activity on Tris-acetate-EDTA and Formaldehyde-agarose gels

Asp fl (100 ng) was incubated with 100 µg of substrate RNA (yeast total RNA and t-RNA) in 1 ml of 0.1 M phosphate buffer pH 7.4 for 15 min at 37°C. Electrophoresis of the samples and controls were carried out as described by Maniatis *et al.* [14].

Degradation of RAW cell RNA by Asp fl

Specific ribonuclease activity of Asp fl on eukaryotic ribosomes was investigated by treating RAW cells with Asp fl followed by extraction of RNA from treated cells. Equal number of RAW cells (5 × 10⁹ cells in 5 ml of medium as described in cell lines) in two culture flasks were incubated at 37°C in a 5% CO₂ incubator for 2 h. The medium was aspirated and cells in one flask were treated with Asp fl (80 ng/ml) in the medium with no foetal calf serum (FCS). Medium alone (with no FCS) was added to the control culture flask. The culture flasks were incubated at 37°C in 5% CO₂ incubator for 8 h. Extraction of RNA was carried out using a single step method of Chomczynski and Nicoletta [15]. The aliquots of total RNA samples were run on a formaldehyde gel as described above.

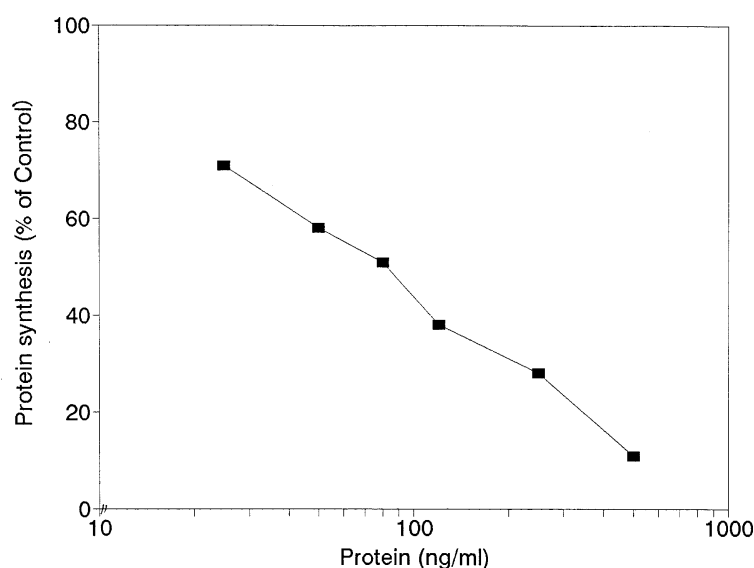


Fig. 1. Percentage inhibition of protein synthesis in RAW cells at various concentrations of Asp fl. RAW cells were incubated with various concentrations of Asp fl for 8 h followed by incubation with ^{14}C labelled leucine for 12 h. Cellular proteins were precipitated with TCA and their radioactivity was measured in a beta counter.

Chemical modification of Asp fl by DEPC and Iodoacetamide

Histidine residues of Asp fl were modified by incubating 10 μg of Asp fl with 0.1% v/v of DEPC at 37°C for 12 h [14]. For alkylation, purified Asp fl (1.0 mM) was incubated with 50 mM iodoacetic acid in 0.1 M sodium acetate buffer pH 5.5 for 18 h at 23°C [16]. Modified Asp fl was evaluated for ribonuclease activity and cytotoxicity.

Cytotoxicity assay

Cytotoxicity assay was carried out using a rapid colorimetric method [9, 17]. Cells were scrapped, centrifuged, resuspended in the fresh medium and dispensed into 96 well plates at a density of 10^4 cells/100 μl /well. On the following day cells were incubated with various concentrations of purified Asp fl and modified Asp fl for 8 h. Antigen was not added in control wells. Cell viability was measured by incubating the cells with 0.5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) in DMEM for 30 min. Medium was aspirated and cells were lysed using 100 μl of acidified isopropanol (40 mM HCl in isopropanol). Absorbance was measured at 570 nm in NUNC ELISA reader. Cytotoxicity assays were performed thrice. Values reported represent the average of three independent readings.

Results

Assay of protein synthesis

Asp fl incubated with RAW cells for 8 h inhibited cellular protein synthesis as measured by radioactivity counts of ^{14}C labelled leucine incorporated into the cellular proteins (Fig. 1). Various concentrations of Asp fl inhibited protein synthesis to an extent of 90% of the control values. IC_{50} of Asp fl was found to be 4.5 nM or 80 ng/ml.

Identification of ribonuclease activity

The ribonuclease activity of Asp fl was demonstrated on agar-yeast RNA gels as indicated by clear region at the site of application (Fig. 2b). DEPC treated autoclaved water as a control showed no activity (Fig. 2e). The approximate ribonuclease activity of Asp fl, in comparison with the standard pancreatic ribonuclease, was found to be 70,000 U/mg (Fig. not shown).

Ribonuclease assay

Ribonuclease activity was determined at each purification step (Table 1). The protein enriched antigenic fraction had a specific ribonuclease activity of 40,000 U/mg while fraction III of phenyl Sepharose exhibited a higher specific activity

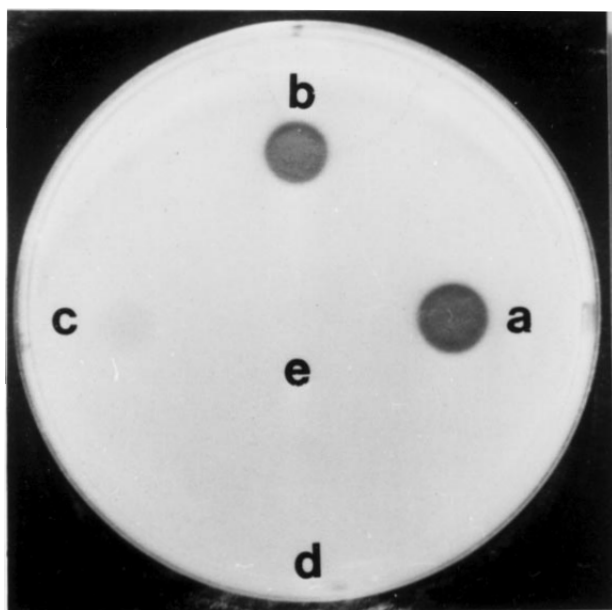


Fig. 2. Ribonuclease activity on agar gel (1%) with yeast RNA (0.2%). (a) Protein enriched antigenic fraction; (b) Purified Asp fl; (c) DEPC modified Asp fl; (d) Alkylated Asp fl; (e) Control. 7.5 μ g protein/10 μ l was applied on the gel and incubated for 18 h at 25°C. Plates were developed with a 0.25% solution of uranyl acetate in 1.25% TCA.

Table 1. Specific ribonuclease activity of various antigenic fractions of *A. fumigatus*

Antigenic fraction	Specific RNase activity (U/mg)
Enriched protein antigenic fraction (crude antigen)	40,000
Fraction III of Phenyl Sepharose column	72,000
Purified Asp fl	100,000

One unit of ribonuclease is defined as the amount of protein required to give a ΔA_{260} 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.

of 72,000 U/mg. Specific ribonuclease activity of purified Asp fl was found to be 100,000 U/mg.

Ribonuclease activity on agarose gels

Yeast total RNA and purified tRNA incubated with Asp fl and standard ribonuclease were electrophoretically analysed on Tris-acetate-EDTA gel (Fig. 3A). Complete disappearance of visible substrate in lanes 3 and 6 indicates that purified Asp fl degraded both total RNA and tRNA. The standard pancreatic ribonuclease was used as a positive control (Lane 2). Lanes 1 and 5 shows the controls (total RNA and tRNA) treated in similar manner.

Figure 3B shows the ribonuclease activity of Asp fl on formaldehyde-agarose gel. It confirms the result obtained by Tris-acetate-EDTA agarose gel and further indicates that Asp

fl acts on all species of RNA as shown by disappearance of various subunits which are visible in the control lanes.

Degradation of RAW cell RNA by Asp fl

We examined the ribosomal RNA in both control and Asp fl treated cells. There was no detectable degradation of 28S and 18S rRNA from untreated cells (Fig. 4, lane B). However, Asp fl treated cells showed five fragments indicating a degradation of ribosomal RNA by Asp fl (Fig. 4, lane A). A 400-base fragment which may be similar to the alpha-fragment produced by alpha-sarcin was also observed (Fig. 4, lane A).

Chemical modification of Asp fl

Asp fl exhibits ribonuclease activity as suggested by results of above experiments. It was chemically modified to alter its enzymatic activity. Modification of histidine residues of Asp fl (8 residues at positions 34, 35, 49, 81, 91, 103, 136 and 149) with diethyl pyrocarbonate resulted in reduced ribonuclease activity of Asp fl (Fig. 2c). Specific ribonuclease activity of DEPC treated Asp fl was found to be 1000 U/mg. DEPC treated Asp fl when examined for its cytotoxic activity on RAW cells showed no cytotoxic activity (Fig. 5). Alkylation of cysteine residues (4 at positions 5, 75, 131 and 147) at their carboxy groups with iodoacetamide resulted in almost total loss of ribonuclease activity as seen on the agar-RNA gel (Fig. 2d). Specific ribonuclease activity of alkylated Asp fl was found to be 780 U/mg. However, alkylated Asp fl did not show any cytotoxicity (Fig. 5).

Discussion

The results show that Asp fl inhibits protein synthesis in the eukaryotic cells. A concentration of 80 ng/ml of Asp fl was required for 50% inhibition of protein synthesis in eukaryotic cells while a concentration of 20 ng/ml of Asp fl has been reported to inhibit 90% of protein synthesis in rabbit reticulocyte lysates (cell free systems) [6]. This difference may be due to limited uptake of Asp fl by eukaryotic cells. Fifty percent of the cultured cells were killed by Asp fl at a concentration of 100 ng/ml as determined by MTT assay [9]. The amount of Asp fl required for cytotoxicity is similar to the amount required for protein synthesis inhibition suggesting that mechanism of Asp fl cytotoxicity could be inhibition of protein synthesis.

Asp fl possesses a specific ribonuclease activity of 100,000 U/mg which is comparable to the activity of T2 ribonuclease isolated from *Aspergillus oryzae* (a Sigma product). The formaldehyde-agarose gel electrophoresis showed that Asp

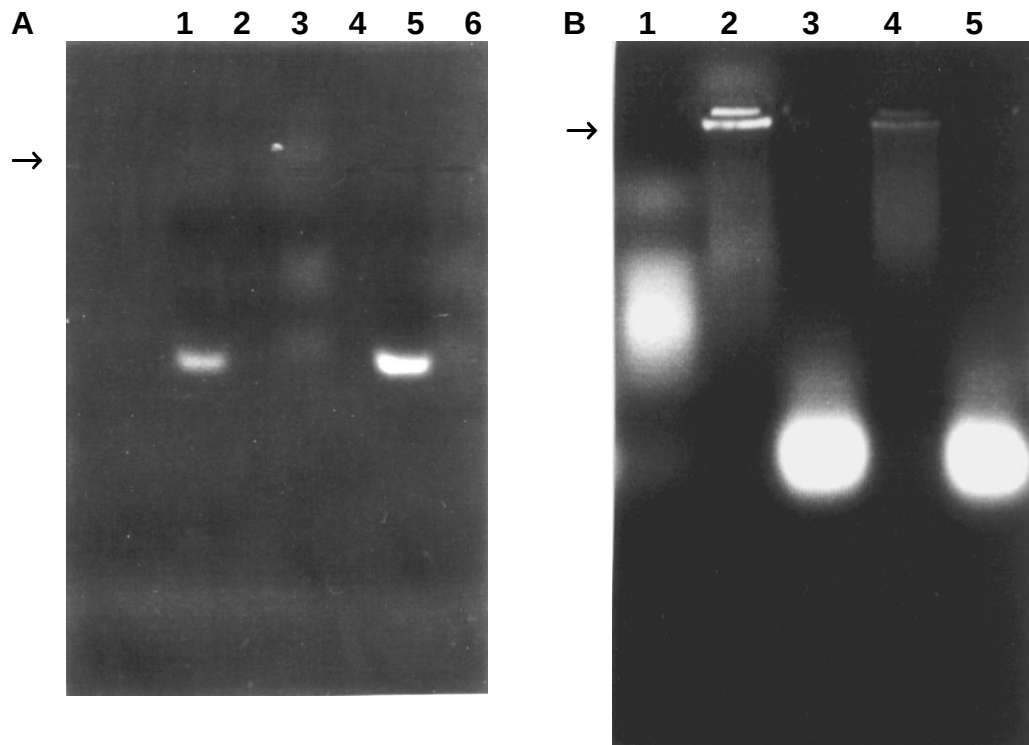


Fig. 3. Ribonuclease activity of Asp fl on (A) Tris acetate agarose gel. Lane 1: total RNA (control); Lane 2: total RNA treated with pancreatic RNase; Lane 3: total RNA treated with Asp fl; Lane 4: empty lane; Lane 5: t-RNA control; Lane 6: t-RNA treated with Asp fl. (B) Formaldehyde agarose gel. Lane 1: total RNA (control); Lane 2: total RNA treated with Asp fl; Lane 3 and 5: t-RNA control; Lane 4: t-RNA treated with Asp fl.

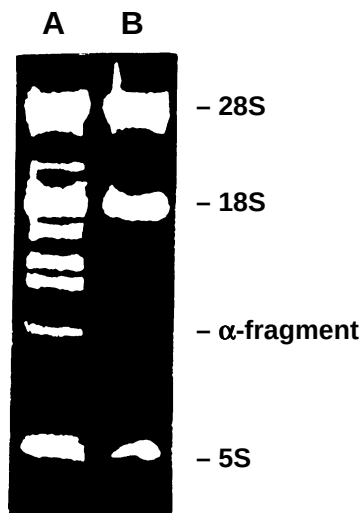


Fig. 4. RNA degradation in RAW cells by Asp fl. RAW cells were cultured in culture flasks with equal number of cells and treated as described in Materials and methods. After 8 h cells were washed twice with phosphate buffered saline and total RNA's were extracted by a single-step purification. Aliquots of recovered RNA's were loaded on a formaldehyde-agarose gel. Lane A: total RNA's from Asp fl treated RAW cells; Lane B: total RNA's from untreated cells.

fl degrades all species of naked RNA, similar to the ribonuclease activity of alpha-sarcin which extensively digests naked RNA by cutting at 3' end of every adenine and guanine irrespective of the single or double stranded nature of the substrate [11]. When the substrate is intact ribosome, Asp fl produced five fragments indicating multiple cleavage sites. However, the earlier reports of rabbit reticulocyte lysate on treatment with Asp fl produced only one fragment of 400 bp similar to the alpha fragment of alpha-sarcin [10, 11]. The five fragments include one fragment of 400 bp approximately which may be similar to the earlier reported alpha-fragment.

Presence of ribonuclease activity of Asp fl suggests that protein synthesis inhibition and cytotoxic activity of Asp fl might be contributed by ribonuclease activity. Amino acid sequence of Asp fl shows presence of four cysteines with 2 disulfide bridges and eight histidine residues [7], which are reported to be important for ribonuclease activity of fungal ribotoxins [18]. Modification of histidine and cysteine residues of Asp fl reduced its enzymatic activity similar to restrictocin and mitogillin [19]. The present study establishes that cysteine and histidine residues are essential for ribonuclease activity of Asp fl. Further, it confirms the role of ribonuclease activity in cytotoxicity of Asp fl similar to other cytotoxic ribonucleases [19].

Use of fungal toxins such as alpha-sarcin, restrictocin and

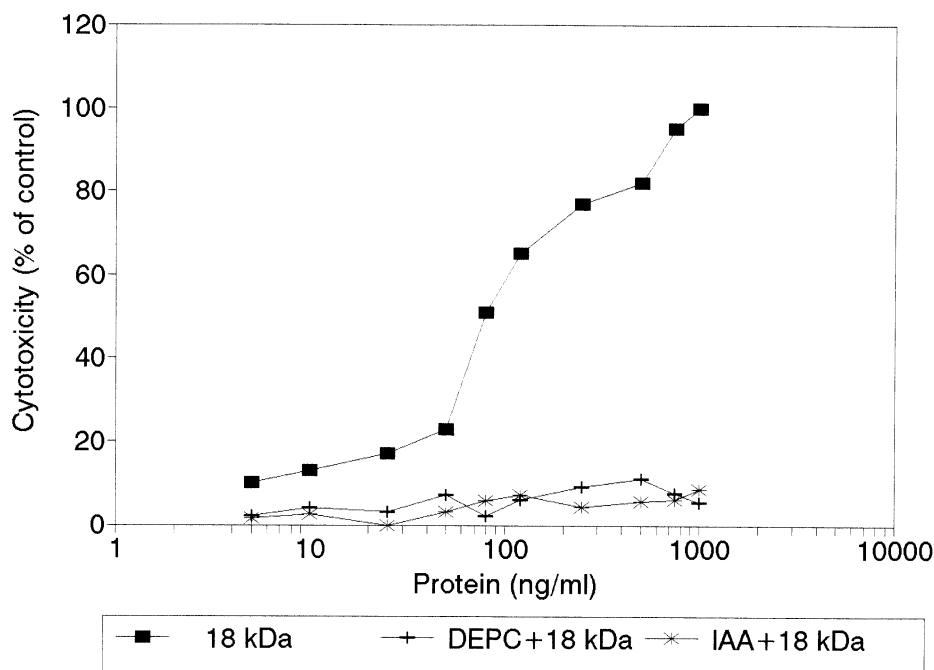


Fig. 5. Cytotoxicity of various concentrations of modified Asp fl and Asp fl to RAW cells. (A) Asp fl; (B) DEPC treated Asp fl; (C) Alkylated Asp fl.

mitogillin for cancer chemotherapy has remained limited on account of hypersensitivity reactions in the patients during clinical trials [20]. Epitope mapping of Asp fl has shown presence of a major B and T cell epitope which has both IgG and IgE binding activity [21].

Elimination of immunoreactive domains from Asp fl may result in a cytotoxin with reduced hypersensitivity. It is interesting to note that Asp fl from the pathogenic species *Aspergillus fumigatus* is functionally similar to ribotoxins of other nonpathogenic species of *Aspergillus* genus. It might be due to existence of similar genes in various *Aspergillus* species and some mutations might have led to the variation in the protein sequence. Further, it suggests that mere presence of a gene encoding for a protein functionally similar to Asp fl does not make an *Aspergillus* species pathogenic. The above fact has also been established in an earlier study showing no loss of pathogenicity in double mutants of Asp fl and alkaline protease [22]. Thus, indicating that Asp fl is one of the various important virulent factors of *Aspergillus fumigatus*. To conclude, this investigation established that Asp fl possesses significant ribonuclease activity which is essential for its cytotoxicity.

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