

Expression of an epitopic region of Asp f I, an allergen/antigen/cytotoxin of *Aspergillus fumigatus*

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Abstract

The gene for an 18 kD allergen/cytotoxin of *Aspergillus fumigatus* was cloned in pUC-19 vector and expressed in *Escherichia coli* JM109. Digestion of this gene with AluI resulted in four fragments of 216bp, 120bp, 39bp and 21bp. These fragments were cloned in the Sma-I site of pUC-19. The recombinants thus, generated after transformation in *E. coli* JM109, were screened using monoclonal antibodies raised against the Asp f I. The fusion protein containing 120 bp AluI fragment was recognised by the MoAb indicating presence of epitope(s) in the 120 bp region. The study indicates a viable strategy for identification and expression of an immunologically active domain of a major allergen/antigen of *A. fumigatus* for the first time. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Members of the genus *Aspergillus* are considered as opportunistic pathogens and are responsible for a variety of allergic and invasive forms of Aspergillosis. Among various species of *Aspergillus fumigatus* is regarded as the major causative agent of Aspergillus induced infections in humans. *A. fumigatus* secretes a number of biologically active allergens/antigens which interact with the host and produce a wide clinical spectrum of Aspergillus infections. Specific antibodies raised in the host to these immunodominant proteins/glycoproteins have diagnostic significance and may also play a role in pathogenesis [1]. Identification of functional domains and epitopic sequences would offer a better understanding on the mechanism of pathogenesis of aspergillosis and also for the development of diagnostic technologies based on synthetic epitopes.

As a result of the advent of recombinant DNA technology, various allergens/antigens of *A. fumigatus* are now being expressed and are available for charac-

terisation [2–6]. The first recombinant *A. fumigatus* allergen, Asp f I, a major allergen/antigen, is demonstrated to have ribonuclease activity and is cytotoxic to eukaryotic cell lines. [7,8]. Diagnostic significance of Asp f I has been established as presence of specific IgE antibodies to Asp f I in 85% of the allergic aspergillosis patients was observed [9]. Secretion of Asp f I in the urine of invasive aspergillosis patients has also been reported [10].

In view of its multifunctional nature and importance in pathogenesis and diagnosis, molecular characterisation of Asp f I is necessary with respect to its immunodominant regions and catalytic sites. In the present study, efforts have been made to identify, express and characterise the epitopic regions of Asp f I.

2. Materials and methods

2.1. Fungal cultures and RNA extraction

Aspergillus fumigatus was grown in synthetic media as described earlier [11]. Four day old cells were used to isolate the total RNA. The cells were suspended in 10 mM Tris–HCl pH 7.5 and were subjected to chitinase

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(Sigma, St. Louis, MO) treatment for 10 min at 25°C followed by three cycles of microwave treatment of 3 s each. The reaction was stopped by keeping the mixture in boiling water bath for 5 min. The RNA from these cells was released using RNA extraction buffer (REB) (10 mM Tris–HCl pH 8.6, 1% v/v NP-40, 1.5 mM MgCl₂, 1 mM DTT, 10 mM EDTA, 20 mM Vanadylribonucleoside complex and 10 mM NaCl) in 1:2 (v/v) at room temperature and finally the RNA was extracted with a mixture of Phenol–CHCl₃–Isoamylalcohol (25:24:1v/v/v). The aqueous phase was precipitated with equal volume of ice cold iso-propanol and dissolved in 100 µl of diethyl pyrocarbonate (DEPC) treated distilled water.

2.2. Formaldehyde gel electrophoresis

The extracted RNA from *A. fumigatus* was analysed on 1.3% formaldehyde gel electrophoresis [12].

Primers:

US1: –5'TGGACATGTATAAACCAG3'–

US2: –5'ATGAGAACACAGTCTCAAGTC3'–

primers used in the present study were as reported by Bir et al. [13].

2.3. Reverse transcription and polymerase chain reaction

RNA (1 µg) was dissolved in DEPC treated water heated to 90°C for 5 min and rapidly chilled on ice. The total cDNA against the *AspfI* was constructed using TaqDNA polymerase and US1 oligonucleotide primer [14]. Ten microliters of the above step was used as template for the isolation of the cDNA coding for *AspfI*. (10 µl of 10 × PCR buffer, 2.5 mM MgCl₂, 200 µM of dNTP, 250 ng of both US1 and US2). Oligonucleotide primers, three units of TaqDNA polymerase were used in the reaction mixture. The reaction mixture was subjected to amplification for 30 cycles. (90°C for 1 min, 48°C for 1 min, and 72°C for 3 min).

2.4. Agarose gel electrophoresis

The amplified DNA was observed by running 1% gel electrophoresis in TBE system [12].

2.5. Cloning of the cDNA in pUC-19

The cDNA thus formed was eluted from the agarose gel [12] and the DNA was cloned in the Sma-I site of pUC-19. The ligation mixture (20 ng) was transformed in *E. coli* JM109 [12]. The transformants were screened on LB agar plates that contained 100 µg ml^{–1}, Ampicillin, 40 µg ml^{–1} X-gal, 80 µg ml^{–1} IPTG [12].

2.6. Immunochemical screening of the clones

Positive clones were screened using MoAb raised against the *AspfI* gene [15].

2.7. Expression of *AspfI* gene

The positive clone pUS3-8 screened in the above step was grown in 50 ml of LB broth containing 100 µg ml^{–1} Ampicillin, 40 µg ml^{–1} X-gal and 80 µg ml^{–1} IPTG for 6 h in an environmental shaker at 300 rpm at 37°C [16]. The growth was stopped by keeping the cells in ice for 30 min. The cells were harvested by centrifuging at 20 000 × *g* for 10 min at 4°C and washed with 50 mM Tris–HCl pH 7.5. The cells were sonicated twice at 50 Hz frequency for 3 min in ice, the disrupted cells were then centrifuged at 100 000 × *g* for 120 min to remove the membrane fraction and the supernatant was used to identify the fusion protein.

2.8. Cloning of the immunodominant domain of the *AspfI* gene

About 0.5 µg of the *AspfI* gene was digested with AluI enzyme (in view of presence of three AluI sites in the *AspfI* gene), and the fragments were resolved on 5.5% polyacrylamide gel electrophoresis [12]. All the fragments of *AspfI* gene were cloned in the Sma-I of pUC-19 using T4 DNA ligase. From the recombinants, the positive clones were identified with monoclonal antibodies (1:200) MoAbs raised against the *AspfI* gene. Each positive clone was expanded in 10 ml of LB media that contained 50 µg ml^{–1} of ampicillin and the recombinants were isolated. The recombinant plasmids thus obtained were digested with EcoRI and BamHI.

2.9. Isolation of the fusion protein

The recombinant plasmid pUS-8 that contained the 120bp fragment was grown in a 50 ml of LB media with 50 µg ml^{–1} of ampicillin, 80 µg ml^{–1} IPTG and 40 µg ml^{–1} of X-gal [16] for 6 h at 37°C at 300 rpm in an environmental shaker (Labline, USA). To isolate the fusion protein from the culture, the cells were then pelleted at 20 000 × *g* at 4°C for 10 min. The cells were then suspended in 2 ml of 50 mM Tris–HCl pH 8.0 and sonicated at 50 Hz at 0°C, cell debris and other components were separated at 34 000 × *g* at 4°C for 10 min in L7-35 Ultracentrifuge, Beckman. In order to get the cytosolic fraction the supernatant was further centrifuged at 100 000 × *g* at 4°C for 120 min in L7-35 Ultracentrifuge, Beckman.

2.10. ELISA

Immunoreactivity of the cytosolic fraction containing the fusion protein was examined with 1:200 diluted monoclonal antibodies against Asp fI by indirect ELISA [17]. Native purified Asp fI protein and cytosolic fraction of *E. coli* JM 109 without the recombinant plasmid pUS3-8 or pUS-8 were taken as positive and negative controls, respectively, to identify the fusion protein.

2.11. Immunoblot analysis of the clone

The cytosolic fractions of pUS3-8 was separated on 10% SDS-PAGE. While clone pUS-8 was separated on 15% SDS-PAGE [18] and electrotransferred on to nitrocellulose membrane [19]. The immunoreactivity of fusion protein was detected using the MoAb (1:200) raised against Asp fI.

3. Results

3.1. Isolation of *Asp fI* gene

From the four days old clinical isolate of *A. fumigatus* the total RNA was isolated. The total RNA when extracted with phenol saturated with DEPC treated water followed by CHCl_3 : Isoamylalcohol (24:1) yielded $2 \mu\text{g} \mu\text{l}^{-1}$ of total RNA from 500 mg of cells. Electrophoresis of this RNA in 1.1% formaldehyde gel gave highly pure form of total *A. fumigatus* RNA (Fig. 1(A)). Using US1 and US2 primers the *Asp fI* gene was

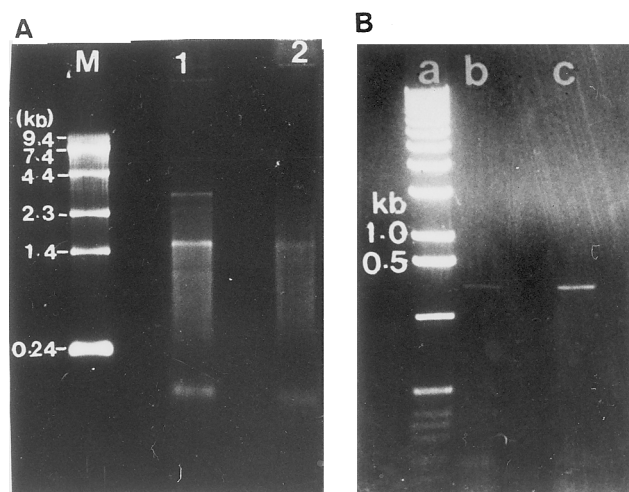


Fig. 1. (A) one point one percent formaldehyde gel electrophoresis of total RNA extracted from the *A. fumigatus*. Lane M; RNA Molecular size markers (GIBCO BRL); lane 1, 5 μg of the total RNA was loaded; and lane 2, 2.5 μg of the total RNA was loaded. (B) Isolation of *Asp fI* gene of *A. fumigatus* using RT-PCR. Lane a, 100 bp ladder; lane b, 10 μl of RT-PCR product; and lane c, 20 μl of RT-PCR product.

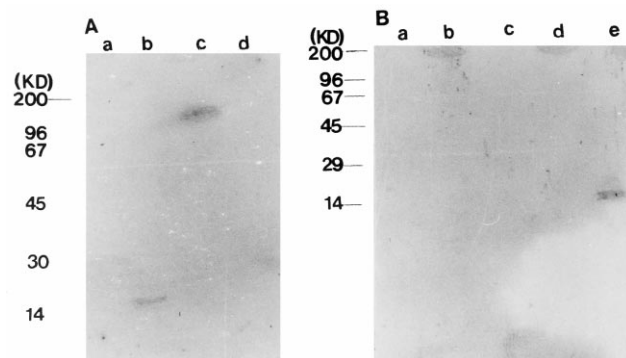


Fig. 2. (A) Immunoblot analysis of the cytosolic fraction of clone pUS3-8. Lane a, cytosolic fraction of *E. coli* JM 109 cells; lane b, native Asp fI protein isolated from *A. fumigatus*; lane c, cytosolic fraction of clone pUS3-8; and lane d, cytosolic fraction of *E. coli* JM 109 containing pUC-19 plasmid. (B) Immunoblot analysis of the cytosolic fraction of clone pUS-8. Lane a, cytosolic fraction of *E. coli* JM 109 cells; lane b, and d, cytosolic fraction of clone pUS-8; lane c, cytosolic fraction of *E. coli* JM 109 containing pUC-19; and lane e, native Asp fI protein isolated from *A. fumigatus*.

isolated by reverse transcript polymerase chain reaction (RT-PCR) (Fig. 1(B)). The PCR product was electrophoretically separated on agarose gel and eluted from the gel, and was then further cloned in the Sma-I of pUC-19. The ligation mixture was successfully transformed on to *E. coli* JM 109 with a transformation efficiency of 1×10^8 cells μg^{-1} of DNA. The recombinants were screened from non-recombinants using X-gal, IPTG and ampicillin. From these recombinants, positive clones were screened using (1:200) monoclonal antibodies against Asp fI (Fig. 2(A)). Out of several positive clones pUS3-8 was picked up and the presence of the insert was checked by performing PCR using forward and reverse primers of M13mp19 which successfully amplified the gene (480 bp).

3.2. Identification of the immunodominant epitope

The pUS3-8 that contained the *Asp fI* gene was amplified by PCR using forward and reverse primers of M13mp19, the amplified product was digested with AluI and cloned in the Sma-I of pUC-19 (Fig. 3). The ligation mixture was successfully transformed on to *E. coli* JM 109 with a transformation efficiency of 1×10^8 cells μg^{-1} of DNA. The recombinants were screened from non-recombinants using X-gal, IPTG and ampicillin. From these recombinants positive clones were screened using (1:200) monoclonal antibodies raised against Asp fI. The positive clone pUS-8 that contained an insert of 120bp was identified by digesting the pUS-8 with EcoRI and BamHI and performing PCR using forward and reverse primers of M13mp19 (Fig. 4). At $80 \mu\text{g} \text{ml}^{-1}$ concentration of IPTG the fusion protein was expressed in *E. coli* JM109 when grown for 6 h at 37°C , the cytosolic fraction that contained the fusion

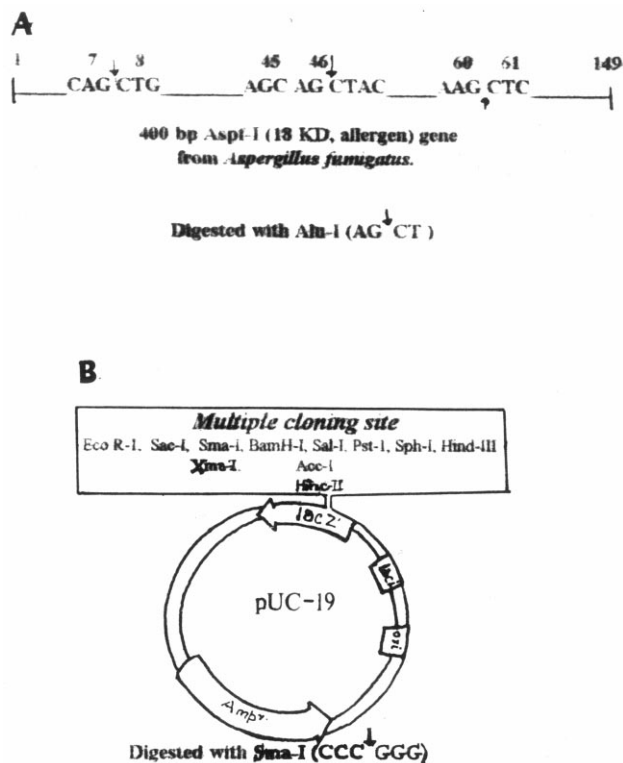


Fig. 3. Schematic representation of the cloning strategy for the identification of the immunodominant region of the *Asp f I* gene.

protein reacted with the monoclonal antibodies against the native *Asp f I* in immunoblot (Fig. 2(B)) and in ELISA (Table 1).



Fig. 4. Identification and confirmation of the 120 bp *AluI* fragment of *Asp f I* gene in pUC-19. Various pUS clones obtained after cloning the *AluI* digested fragments of *Asp f I* gene in the *Sma-I* of pUC-19 were subjected to *BamHI* and *EcoRI* digestion. Lane 1, Intact pUC-19 molecule; lanes 2–6, clones pUS-5, 6, 7, 8, and 9; lanes 7–10, clones pUS -1, 2, 3, and 4; and lanes 11–12 are clone pUS 3-2 and 3-1.

Table 1

ELISA absorbance values for immunoreactivity of recombinant fusion epitope with monoclonal antibody against native antigen *Asp f I*^a

With recombinant fusion epitope	With Native <i>Asp f I</i>	With synthetic epitope (11 amino acid peptide)
0.850 ± 0.049	0.997 ± 0.030	0.346 ± 0.056

^a All the readings are mean values representing three readings for each sample.

4. Discussion

In view of increasing incidence of fungal diseases, the necessity for fungal diagnostics and therapeutics is being felt all over the world. Unless the nature of antigens relevant for diagnosis, protection and pathogenesis are well understood, it is not possible to develop diagnostics or therapeutics for any infectious disease. Lack of availability of purified *Aspergillus* antigens for diagnostic testing resulted in non availability of any international reference standards or WHO reference standards.

Reports on identification of epitopes of immunodominant allergens/ antigens of *A. fumigatus* are limited [20–22]. General methods applicable for such studies for proteins include crystallographic analysis, chemical and enzymatic modifications, and synthesis of overlapping peptides. The first two methods require large amounts of purified antigens in native form. The third method is relatively simple but poses limitations as peptides of certain sizes do not attain proper folding which is necessary for conformational epitopes to bind with the antibody. In the present study, a recombinant approach was adopted to identify the epitopic regions of a major allergen/antigen of *A. fumigatus* by digestion of *Asp f I* gene with *AluI* followed by expression of the fragments as fusion proteins. Recombinant expression of epitopic domains of human hsp60 and HIV Rev protein has been reported using a synthetic oligonucleotide coding for the epitope [23,24]. However, use of restriction enzyme digestion for epitope mapping has been used in the current report.

The total RNA of *A. fumigatus* has been isolated by a modified protocol which yielded good quantities of RNA in reasonably pure form. Chitinase and microwave treatment efficiently hydrolysed the cell walls of *A. fumigatus*. RE buffer, the buffer containing 1% Nonidet P-40 and 20 mM of vanadyl ribonucleoside complex (a potent RNase inhibitor), totally solubilized the membranes resulting in 2 µg of RNA per mg of cells.

The fusion protein expressed by the clone containing 120 bp *AluI* fragment was recognised by the MoAb indicating presence of epitope(s) in the 120 bp region (aa residues from 8–47). In ELISA, the reactivity of the fusion peptide was less than the native *Asp f I* while it

was significantly higher than the eleven amino acid synthetic peptide from the same region (sequence as in patent [22]). This may be as a result of the difference in conformational folding of a short peptide (only 11 amino acids) and fusion protein with epitopic domain (40 amino acids). Epitopes of Aspfl have been mapped by three other groups using different strategies such as T-cell clone mapping, immunisation of mice with overlapping synthetic peptides or immunoreactivity analysis of the synthesised peptides by ELISA and immunoblot using sera of aspergillosis patients. Kurup et al., have shown the presence of epitopes in five synthetic peptides of Aspfl [20]. One of the epitopic peptides with amino acid residues 1–15 identified by Kurup et al., and an epitopic region (1–20 aa residues) identified by Chauhan et al., 1997, by mapping T-cell specific clones, correspond to the same region identified in the present study (8–47 aa residues) [20,21]. These observations confirm the presence of epitopic region in the N-terminal region of Aspfl. Based on computer algorithms using hydrophilicity, mobility, etc., few epitopic regions of Aspfl were predicted. All the ten programmes used indicated N-terminal region as one of the probable immunodominant domains. Further, few overlapping synthetic peptides from the same region were able to inhibit 89% of the *A. fumigatus* specific IgG antibody binding by the native protein [22]. The current study confirms the location of a 39 amino acid immunoreactive domain from the 5' end of the *AspfI* gene.

Recombinant and synthetic epitopes have been employed in the preparation of vaccines and diagnostic technologies for infectious diseases such as HIV virus, Influenza virus, Hepatitis virus, Malaria parasite and for allergens such as milk allergen, and Birch pollen allergen [25]. Development of vaccines for fungal infections may be possible with identification and recombinant expression of certain sequential domains which will stimulate the production of Th1 type of cytokines. Complex antigenic architecture of fungus may not be a limiting step anymore for development of fungal diagnostics and vaccines in near future.

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