



Molecular and immunological characterization of subtilisin like serine protease, a major allergen of *Curvularia lunata*

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ABSTRACT

Serine protease from numerous sources have been identified and characterized as major allergens. The present study aimed to clone, express and characterize a serine protease from *Curvularia lunata*. cDNA library screening identified partial protease clones. A clone showed significant homology to subtilisin like serine proteases from *Aspergillus* and *Penicillium* species. Full length sequence was generated by RACE PCR, subcloned in pET vector, protein expressed in *Escherichia coli* and purified from inclusion bodies yielding 0.5 mg/L of culture. Bioinformatic analysis identified serine protease motifs of subtilase family, catalytic triad and N-glycosylation sites on the primary sequence. The protein resolved at 54-kDa on SDS-PAGE and was recognized as a major allergen on immunoblot with 13/16 *C. lunata* sensitive patients' sera in ELISA and immunoblot. Recombinant protein reacted with rabbit polyclonal antibodies against alkaline serine proteases from *C. lunata*. Recombinant protein required 50–56 ng of same protein for 50% inhibition of IgE binding in competitive ELISA. In addition, 13 of 16 patients' samples showed significant basophil histamine release upon stimulation with purified recombinant protein. In conclusion, a 54 kDa major allergen of *C. lunata* was cloned, expressed, characterized and showed biological activity. It has potential to be used in molecule based approach for allergy diagnosis and therapy.

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Introduction

Fungal allergens play an important role in the pathogenesis of allergic airway inflammation and asthma. Among asthmatics, mold allergy affects 70% of adults and 45% of children (Simon-Nobbe et al., 2008). *Curvularia lunata* belonging to class Deuteromycetes is an inhalant allergen implicated in allergic rhinitis, asthma and allergic bronchopulmonary mycoses (Corey et al., 1997; Elliott and Taylor, 1997; Galant et al., 1998). Skin tests with *C. lunata* extract exhibited sensitization in 7–26% of atopic patients in different countries (Platts-Mills and Chapman, 1991; Gupta et al., 1999; Chou et al., 2002; Wiedermann, 2005). IgE binding studies with different extracts of the mold demonstrated 10–14 aller-

genic proteins (Gupta et al., 1999). *C. lunata* showed a close antigenic/allergenic relationship among *Curvularia* species and with other fungal genera namely *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum* and *Fusarium* (Gupta et al., 2002a, b). Previously, a 31 kDa serine protease was identified as major allergen and purified to homogeneity from *C. lunata*. Subsequently, enolase and cytochrome c allergens were identified and produced as recombinant proteins that offer scope for component resolved diagnosis and therapy. (Gupta et al., 2004; Sharma et al., 2006, 2008).

Proteolytic enzymes from fungi were implicated in IgE mediated reactions (Simon-Nobbe et al., 2008). A study showed that serine protease levels in the environment were linked to incidence of asthma (Montealegre et al., 2004). Alkaline and vacuolar serine proteases were major allergens of fungi such as *Aspergillus*, *Cladosporium*, *Penicillium*, *Rhodotorula* and *Trichophyton* (Pöll et al., 2009). Proteolytic activity increases bronchial epithelial permeability by degrading proteins of desmosomes, tight and adherens junctions and thereby facilitating antigen access to the immune system (Robinson et al., 2001). Fungal serine proteases such as Asp fl 1, Cla c 9, Cla h 9, Pen ch 13, Pen ch 18 and Pen o 18 were cloned and expressed as recombinant proteins (Yu et al., 1999; Shen et al., 2001, 2003; Chou et al., 2002, 2008; Pöll et al., 2009). These homogeneous preparations can be modified by reducing allergenic activity to foster advantageous immunologic properties like abrogating IgE

Abbreviations: BLAST, basic local alignment search tool; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IUIS, international union of immunological societies; LB, Luria broth; Ni-NTA, nickel-nitrilotriacetic acid; PBS, phosphate buffered saline; RACE, rapid amplification of cDNA ends; SDAP, structural database of allergenic proteins; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS, Tris buffered saline.

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binding epitopes or tagging novel sequences for obtaining better immunotherapeutic response.

The present study was aimed to clone, express and characterize serine protease, a major allergen of *C. lunata*. Immunoscreening of *C. lunata* cDNA library identified partial protease clones. A full length cDNA nucleotide sequence of the gene was determined and further subcloned in pET vector. It was expressed as recombinant protein in *Escherichia coli*, purified and characterized by bioinformatic and immunological methods using hypersensitive patients' sera.

Materials and methods

Immunoscreening of cDNA library with *C. lunata* allergic patients' sera

Screening of *C. lunata* cDNA library was performed using *E. coli* preadsorbed *Curvularia* sensitive patients' pooled sera 1:10 (v/v) in Tris buffer saline (TBS). Briefly, the phages containing cDNA library were used to infect *E. coli* XL-1 Blue cells, plated on 90 mm New Zealand yeast agar plate (density 300–400 plaques/plate) and incubated at 42 °C for 4 h. The nitrocellulose disc soaked in 10 mM isopropyl-beta-thiogalactopyranoside was overlaid on the plate and incubated overnight at 37 °C. Subsequently, membranes were washed with TBS, blocked with 3% defatted milk at 37 °C for 1 h, and incubated with preadsorbed pooled patients' sera overnight at 4 °C. The overlaid membranes were washed with TBS-Tween 20 (0.05%) and incubated with antihuman IgE peroxidase (Sigma, 1:1000) at 37 °C for 2 h. The positive clones were verified by secondary screening. Plaques were excised using ex-assist helper phage to get phagemid as DNA template for PCR sequencing.

cDNA cloning and sequencing

Total RNA was isolated from 4-day-old *C. lunata* culture by EZ-RNA total RNA isolation kit (Biological Industries, Israel). The rapid amplification of cDNA ends (RACE) was employed to deduce full length cDNA sequence coding for subtilisin like serine protease using Roche 5'/3' RACE cDNA amplification kit (Roche Applied Science, USA). For 5'-RACE, first strand of cDNA was synthesized using gene specific antisense primer SP1 (5'-CGTTGTGCCAGTGTCAATGAC-3') and total RNA as template source followed by 3' poly (A) tailing using dATP and recombinant terminal transferase. The product was further amplified by normal PCR using SP1 (5'-CGTTGTGCCAGTGTCAATGAC-3') and anchored oligo-dT (5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTIV-3' V=A,C or G) primer AP 1. For 3'-RACE, first strand was synthesized from total RNA using Oligo dT-anchor primer (5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTIV-3' V=A,C or G) which was further amplified using gene specific sense primer SP 2 (5'-ACG TCT ATG CCG TCA AGG TCC-3') and PCR anchor primer AP2 (5'-GACCACGCGTATCGATGTCGAC-3'). RACE products were purified and sequenced. The primers were designed for full length sequence SP3 (5'-CGAGGCTCACTATCCCCTTCC-3') and SP4 (5'-CTCGAGGTGCTAGAGCTTGCCA-3') and cDNA was synthesized from total RNA and sequenced.

Sub-cloning in pET22b+ expression vector

The primers SP5 (5'-TTGAGCTGGAATTCGATGAAGTACAGCCTG-ATCCCGCG-3') and SP6 (5'-ATAGGCCAAAGCTTGAGCTTGCCACCG-ACGTGGTGAG-3') were used to amplify cDNA fragment with *Eco* R I and *Hind* III sites, respectively at flanking ends. The PCR product was purified using Qiagen kit. The PCR product and pET22b+ were digested with *Eco* R I and *Hind* III restriction enzymes and ligated using T4 DNA ligase at 16 °C overnight. The ligation mix was transformed into *E. coli* DH5 α cells and selected on Luria broth (LB)

ampicillin plates. The positive clones obtained were confirmed by restriction digestion and PCR amplification. The plasmid containing this cDNA fragment was designated as pCL3.

Computational analysis of sequence and homology search

The ORF and deduced protein obtained from the nucleotide sequence were analyzed using DNASTAR software for molecular weight and pI. The homology search was carried out using CLUSTAL-W and basic local alignment search tool (BLAST) algorithm available at National Center for Biotechnology Information. The protein sequence was analyzed for conserved domain. The allergenicity of the sequence was predicted using Allpred tool and homology search in SDAP database.

Expression and purification of the clone

The plasmid pCL3 was transformed into *E. coli* BL21 cells, grown till O.D. 0.8, and induced with 1.5 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) for 3 h and cells harvested. The cell pellet was suspended in binding buffer (8 M Urea, 100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 8.0), sonicated, and centrifuged at 15,000 rpm for 30 min at 4 °C. The supernatant was loaded onto Ni-NTA resin equilibrated with binding buffer. The his-tagged protein was eluted using 250 mM imidazole in the binding buffer. The eluted fractions were desalted and concentrated using Amicon Ultra-15 centrifugal device (Millipore, USA). Protein concentration was estimated using bicinchoninic acid assay.

SDS-PAGE and immunoblot

SDS-PAGE (12% gel) was performed under denaturing conditions to determine the protein profile of Ni-NTA purified recombinant protein. The gel was stained with 0.1% Coomassie brilliant blue (CBB). For immunoblot, SDS-PAGE-resolved protein was electrophoretically transferred to a nitrocellulose membrane (Towbin et al., 1979) using a mini transblot cell (Bio Rad, CA). The transfer of proteins on membrane was ensured by Ponceau-S (0.1% in 1% acetic acid) staining. IgE binding of purified protein was assessed by incubating the strips with *Curvularia* positive patients' serum pool and individual patients' sera (1:10, v/v in PBS), separately. As a control, strips blotted with purified protein were incubated with control sera.

Zymography

The enzymatic activity of purified protein was checked by gelatin zymography (Heussen and Dowdle, 1980). For this, purified recombinant protein was fractionated on 12% polyacrylamide gel containing 0.1% gelatin as substrate. After electrophoresis, the gel was washed with 25% isopropanol in 10 mM Tris-HCl, pH 7.9, incubated overnight at 37 °C in 100 mM Tris-HCl, pH 7.9, stained with CBB and destained.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis

The purified protein was run on SDS-PAGE and stained with CBB. The stained band/protein was cut using a sterile blade and placed in a fresh eppendorf tube. The MALDI-TOF analysis of the trypsinized sample was carried out at The Centre for Genomic Application, New Delhi. The MALDI TOF mass spectrometer was tested and calibrated using a protein standard form Bruker Daltonics. The protein standard is designed for testing of MALDI-TOF mass spectrometers in a mass range between ~10,000 and 70,000 Da. The result from MALDI was obtained in the form of MASCOT search chart.

5'	11	21	31	41	51	61	71	81	91	
1 1	M K Y ATGAAGTACA	S L I A GCCTGATCGC	A L P CGCGCTCCCG	A L A GCCTTGGCCG	A A S P CTGCGTCCGC	T F S CACCTTCTCG	T E T ACAGAGACGA	I H K Q TTCACAAGCA	S A P GTCTGCCCTT	U L S GTGCTCTCGT
34 101	S T S A CGACGTCCGC	K E U CAAGGAGGTC	P N S CCCAACAGCT	Y M U U ACATGGTCGT	F K K CTTCAAGAAG	H U K CACGTCAAGG	D A S K ACGCTCCAA	H H D GCACCATGAC	W U Q TGGGTCCAGA	S U H S GCGTCCACTC
68 201	K N T CAAGAACACC	Q E R CAGGAGCGCA	M E L R TGGAGTCTGC	K R S AAAGCGCTCC	S D L AGCCATCTGC	P U S N CTGTGAGCAA	E U F CGAGGTCTTT	A G L GCTGGCTTCA	K H T Y ATGCACACATA	E L S TGAGCTGTCC
101 301	G L K GGTCTCAAGG	G Y S G GCTACTCCGG	H F D CCACTTTGAC	D E T GATGAGACGC	L E A I TCGAGGCCAT	R N H CCGAACCCAC	P D U CCCGATGTGC	D Y I E ACTACATCGA	R D S GCGTGACTCC	E U R GAGGTCCGCA
134 401	I L G G TTCTCGGCGG	D E P CGATGAGCCC	E T E GAAACCGAGA	N N S P ACAACCTCCC	W G L CTGGGGTCTT	A R I GCCCGTATCT	S H R D CGCACCGCGA	S L S CTCGCTGAGC	F G T TTCGGCACCT	W N K Y GGAACAAGTA
168 501	L Y A CCTCTACGCT	A D G GCCGACGGTG	G E G U GTGAGGGTGT	D U Y TGACGTCTAC	U I D GTCATTGACA	T G T N CTGGCACAAA	U D H CGTTGACCAC	U D F GTGCACTTCG	E G R A AGGGCCGTGC	K W G CAATGGGGC
201 601	K T I AAGACATCC	P N G D CCAACGCGCA	A D E TGCCGATGAG	D G N GATGGCAACG	G H G T GCCACGGCAC	H C S CCACTGCTCT	G T U GGTACCGTTG	A G K K CCGGCAAGAA	Y G U GTACGGTGTT	A K K GCCAAGAAGG
234 701	A H U Y CACACGTCTA	A U K TGCCGTCAAG	U L R GTCCTCCGCT	S N G S CCAACGGCTC	G T M GGGCACCATG	S D U TCCGATGTGC	U K G U TCAAGGGTGT	E F A CGAGTTTTCG	A K S GCCAAGTCTC	H S E A ACTCCGAGGC
268 801	U S A CGTCTCTGCC	A K N GCTAAGAACG	G K K K GCAAGAAGAA	G F K GGGCTTCAAG	G S T GGCAGCACAG	A N M S CCAACATGAG	L G G CTTGGGAGGT	G K S GGCAAGTCCA	T T L D CCACCCTCGA	M A U CATGGCTGTC
301 901	N A A AACGCCGCCG	U D A G TCGATGCTGG	L H F TCTCCACTTC	A U A GCTGTGGCTG	A G N D CCGGTAACGA	N A D CAACGCCGAC	S C N TCGTGCAACT	Y S P A ACTCTCCAGC	A A E TGCGGCTGAG	N A U AACGCCGTC
334 1001	T U G A CTGTGCGTGC	S T L CTCGACTCTC	L D E TTGGATGAGC	R A Y F GTGCGTACTT	S N Y CTCCAACATC	G K C GGAAAGTGCA	N D I F ACGACATCTT	A P G TGCCCTCTGT	L N I CTCAACTTC	L S T W TCTCCACTTG
368 1101	I G S GATCGGCTCC	K H A AAGCAGCGCA	T N T I CAAAACCCAT	S G T CTCTGGCACC	S M A TCGATGGCTT	S P H I CTCCTCACAT	A G L CGCTGGTCTC	L A Y CTTGCTTACA	M L S L TGCTCTCGCT	Q P A CCAGCTGCT
401 1201	K D S AAGGACTCTG	A Y A U CTTACGCTGT	A D I TGCTGACATC	T P K ACCCCAAGAA	K L K A AGCTCAAGGC	N L I TAACCTGATT	A I G GCTATTGGTA	T U G A CCGTTGGTGC	L S D TCTCTCCGAT	U P S GTTCCAGCA
434 1301	N T A N ACACTGCCAA	U L A CGTCTCGCC	W N G TGAACGGTG	G G S S GTGGTTCCTC	N V T CAACTACACT	D I I GACATCATCG	E K G G AGAAGGGTGG	Y T U TTACACGGTC	K K A AAGAAGGCCG	A S K E CCTCCAAGGA
468 1401	E E K GGAGGAGAG	E S E GAGTCTGAGT	F R I T TCCGCATCAC	I P S CATCCCTCT	L S E CTCTCTGAGC	L E D D TTGAGGATGA	F E K CTTCGAGAG	A K E GCCAAGGAGT	S A G R CTGCTGGCCG	K A H CAAGGCTCAC
501 1501	H U G CACGTCGGTG	G K L # GCAAGCTCTA								

Fig. 1. Complete nucleotide and the deduced amino acid sequence of *Curvularia* subtilisin like serine protease. The sequence consisted an ORF of 1521 nucleotides and 506 amino acids. The conserved domains are presented in box. The dark regions represent potential N-glycosylation sites in the protein sequence.

In vitro testing of purified protein

Blood was collected from patients ($n=16$) showing marked positive skin reactivity (2+ and above) with *C. lunata* extract. Blood was also collected from nonallergic subjects ($n=4$) with negative skin tests to *Curvularia* extract for use as control (C). Serum samples were used for the evaluation of allergenic potency of purified protein by enzyme linked immunosorbent assay (ELISA), ELISA inhibition and immunoblot. Histamine release assay was carried out to determine the biopotency of purified protein.

Specific IgE ELISA

Specific IgE against purified protein was measured in sera of *Curvularia* allergic patients ($n=16$) and controls ($n=4$) by ELISA. *Curvularia* extract and purified protein were coated on a microtiter plate (Nunc ELISA maxisorp, Denmark) at a concentration of 1 $\mu\text{g}/100 \mu\text{L}$ per well and 100 ng/100 μL per well, respectively. The plate was incubated overnight at 4 °C. The unbound antigen was washed out with PBS (0.1 M) and the free sites were blocked with 3% defatted milk in PBS for 1 h at 37 °C. After washing, 100 μL of control sera or individual patients' sera (1:10, v/v in PBS) were added separately to the wells and the plate was incubated overnight at 4 °C. The plate was washed five times with PBST (PBS with 0.05% Tween 20), once with PBS and incubated with 1:1000 (v/v PBS) diluted antihuman IgE peroxidase (Sigma Chemical Co.) for 3 h at 37 °C. Following washing with PBST and PBS, color was developed using o-phenylenediamine in citrate-phosphate buffer, pH 5.0. The

reaction was stopped after 30 min by adding 50 μL of 5 N H_2SO_4 and the absorbance was read at 492 nm.

Specific IgE ELISA inhibition

The serum pool of *C. lunata* positive patients ($n=16$) with high IgE titer was used for ELISA inhibition. The serum (50 μL) was incubated with different concentrations (0.1–1000 ng) of purified protein and *Curvularia* extract separately. Sera of nonallergic subjects were used as control. ELISA was carried out and the values in wells incubated with inhibited serum samples were calculated relative to that of uninhibited serum sample as percent inhibition. Inhibition was presented as percentage decrease in absorbance of sample with inhibitor to that of sample without inhibitor and allergenic potency was defined by establishing the concentration of protein required for 50% inhibition.

Basophil histamine release assay

Peripheral blood was drawn from nonallergic subjects, mixed (1:5, v/v) with 6% dextran in saline containing 0.01 M EDTA and 2% dextrose and incubated for 90 min at 25 °C. The leukocyte-rich upper layer was separated, centrifuged (300 g, 4 °C) and washed two times with saline. A 375- μL sample of cell suspension (2.5×10^6 cells) was taken for each incubation. The basophils in suspension were stripped off bound IgE by incubation with lactic acid buffer for 3.5 min at 37 °C (Kleine Budde et al., 2001). Following incubation, cells were washed with HEPES buffer. Subsequently, the cells were re-sensitized at 37 °C for 90 min with a sensitization mix-

Table 1
Homology with allergens in SDAP database.

S. no.	Allergen	Sequence link in Swissprot /NCBI/ PIR	Sequence length	e score
1	Pen o 18	AAG44478	503	3.2e–124
2	Pen ch 18	AAF71379	494	4.5e–119
3	Pen ch 13	AAF23726	397	8.8e–26
4	Asp f 13	P28296	403	4.9e–21
5	Asp f 1 protease	AAD47202	403	1.3e–19
6	Asp o 13	CAA35594	403	1.3e–19

The link to structural database of allergenic proteins (SDAP) is <http://fermi.utmb.edu/SDAP/>.

Table 2
Homology of translated protein sequence in Swissprot database.

S. no.	Accession no.	Source	Biological nature	Homology
1.	XP.001932152	<i>Pyrenophora tritici</i>	Subtilase type protein precursor	94
2.	ABQ59329	<i>Cladosporium cladosporioides</i>	Vacuolar subtilisin like serine protease	89
3.	CAB45520	<i>Aspergillus fumigatus</i>	Serine protease	85
4.	AAG44478	<i>Penicillium oxalicum</i>	Vacuolar serine protease (Pen o 18)	84
5.	AAD25995	<i>Penicillium citrinum</i>	Alkaline serine protease (pen c 2)	83
6.	AAF71379	<i>Penicillium chrysogenum</i>	Vacuolar serine protease (Pen n 18)	80
7.	BAE44888	<i>Candida albicans</i>	Alkaline serine protease	77

The link to Swissprot database is <http://www.expasy.ch/sprot/>.

ture that contained *C. lunata* patients' sera ($n = 16$) with elevated IgE. Cells sensitized with nonallergic subjects sera ($n = 4$) served as control. The histamine release assay was standardized using a graded amount of protein (1–1000 ng), and the protein concentration inducing optimal histamine release (5 ng) was selected for the assay.

After passive sensitization, basophils were stimulated with *Curvularia* extract or the purified 54-kDa protein (5 ng) in HEPES buffer containing 1 mM CaCl_2 . After 1-h incubation at 37 °C, the reaction was stopped by the addition of 750 μl of ice cold 0.9% NaCl (w/v). After centrifugation, the cell-free supernatant containing histamine was taken in an eppendorf tube and mixed with 200 μl of 12% perchloric acid. The histamine content was determined by the fluorometric method, using o-phthalaldehyde (Shore et al., 1959). Spontaneous histamine release was measured in the supernatant of unstimulated cells. The total histamine content was determined by lysis of cells with 3% perchloric acid (Dhyani et al., 2006). The allergen-induced histamine release was calculated as a percent of the total histamine content after correction for spontaneous release.

Results

cDNA library screening and sequencing of pCL 3

Two clones of approximately 900 bp were identified after screening of library with *C. lunata* allergic patients' pooled sera. The immunoscreening revealed a partial protease clone. Full length cDNA clone of approximately 1600 bp was identified using RACE PCR, sequenced by primer walking method, and subcloned in pET vector to designate as pCL3. The ORF was 1521 bp (accession no. EU622631, Fig. 1) encoding a mature protein of 506 amino acids.

Bioinformatic analysis of protein sequence

The sequence of the protein showed the presence of two conserved motif HGTHCSGTVAG (215–225 residues) and GTSMASPHIAG (379–389 residues, Fig. 1). The motif search by Gene Runner software located HGTHCSGTVAG as serine protease motif of subtilase family with histidine active and GTSMASPHIAG with serine active. The residues forming the catalytic triad are D183, H215 and S381. Two potential N-glycosylation sites have been predicted based on the tripeptide recognition sequence Asn-X-Ser or Asn-X-

Thr. These regions have been highlighted in dark in Fig. 1. Further, the protein sequence has shown significant homology with six fungal allergens namely Pen o 18, Pen ch 18, Pen ch 13, Asp f 13, Asp f 1 and Asp o 3 in SDAP database (Table 1).

Comparison of translated coding sequence of cDNA fragment with NCBI database (Swissprot) showed homology with subtilisin like serine proteases from various sources at both DNA and protein level. At protein level, it was similar to *Pyrenophora tritici* (94%) [XP.001932152], *Cladosporium cladosporioides* (89%) [ABQ59329], *Aspergillus fumigatus* (85%) [CAB45520], *Penicillium oxalicum* (84%) [AAG44478], *P. citrinum* (83%) [AAD25995], *P. chrysogenum* (80%) [AAF71379] and *Candida albicans* (77%) [BAE44888] (Table 2). Theoretical molecular weight was approximately 53.77 kDa with pI of 6.15 demonstrating it to be an anionic protein.

Analysis of recombinant protein and SDS-PAGE

Recombinant protein was expressed in *E. coli* BL21 and yielded 0.5 mg/L of culture. After fractionation of the fusion proteins, recombinant protein of interest was isolated and visualized at 54 kDa on 12% SDS-PAGE and designated as Cur 1 4 by I.U.I.S. Allergen Nomenclature Sub-committee (<http://www.allergen.org/Allergen.aspx>). Further, the recombinant protein was recognized by polyclonal antibody against alkaline serine protease from *C. lunata* on Western blot (Fig. 2).

Proteolytic activity

Sequence homology with Asp f 1, Cla h 9, Pen ch 18 and Pen o 18 allergens showed the presence of a hydrophobic N-terminal leader sequence MKYSLIAALPALAAA followed by a prosequence. The qualitative assay of the purified protein revealed that the protein is not enzymatically active, since there was no clear band on the zymogram (data not shown). It indicates that the protein may be in precursor form like most of the subtilisin like serine proteases.

MS analysis of protein

To investigate the function of 54-kDa protein, it was subjected to tandem mass spectrometric analysis. The peptide sequences were searched in the matrix science database. It showed that *C. albicans*, *P. chrysogenum* and *A. nidulans* are the closest homologs of 54-kDa recombinant protein.

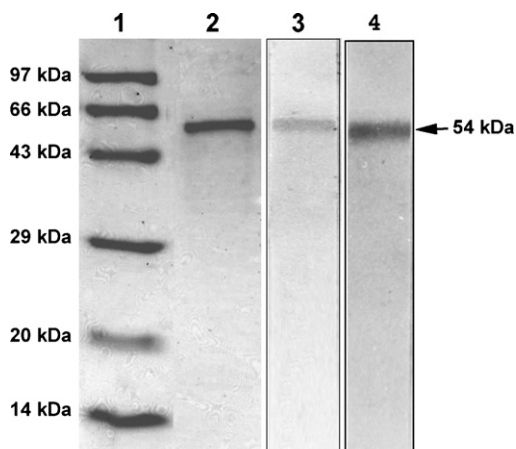


Fig. 2. SDS-PAGE and immunoblot. Ni-NTA purified protein resolved at 54 kDa on 12% SDS-PAGE and probed with *Curvularia* positive patients' pooled sera and anti-Cur I1 by immunoblot. Lane 1 – molecular weight marker, Lane 2 – purified protein, Lane 3 – IgE binding with pooled patient's sera, Lane 4 – binding with anti-Cur I1 polyclonal antibody.

Specific IgE binding of purified protein

Specific IgE binding of recombinant protein was determined by ELISA using *Curvularia* positive patients' sera. ODs ≥ 3 times of controls (average OD: 0.059) were considered as ELISA-positive results. Out of the 16 *Curvularia* positive patients' sera tested (average OD: 0.496), 13 showed elevated IgE binding against 54 kDa protein (average OD: 0.337). Serum samples from patient numbers 3, 12 and 15, however exhibited low IgE binding against purified protein. The 54-kDa protein was recognized by 13/16 (81.25%) of *Curvularia* hypersensitive patients' sera on immunoblot indicating it to be a major allergen (Fig. 3, Table 3).

Allergenic potency of 54 kDa protein

ELISA Inhibition was performed to check the specificity of IgE binding and the allergenic potency (EC_{50}) of the purified recombinant 54-kDa protein. A dose dependent inhibition of IgE binding to solid-phase *Curvularia* extract was observed, when serum pool was preincubated with increasing concentrations (1–1000 ng) of recombinant protein or *Curvularia* extract, separately. The different batches of purified protein (54 kDa) required 50–56 ng of same protein for 50% inhibition of IgE binding (EC_{50}). Here, a maximum of 65% inhibition was achieved with 1 μ g of the purified 54-kDa protein used as an inhibitor for the solid-phase bound *Curvularia* extract. For 50% inhibition of the solid-phase bound *Curvularia*, 172 ng of purified protein was required compared to 48 ng of *Curvularia* extract for the same (Fig. 4).

Table 3

Specific IgE binding of *Curvularia* extract and 54-kDa protein with *Curvularia* hypersensitive patients' sera ($n = 16$).

S. no.	<i>Curvularia</i> extract (OD at 492 nm)	54 kDa recombinant protein (OD at 492 nm)
1	0.531	0.321
2	0.543	0.372
3	0.379	0.128
4	0.465	0.253
5	0.397	0.267
6	0.421	0.268
7	0.54	0.315
8	0.601	0.273
9	0.581	0.45
10	0.526	0.319
11	0.563	0.422
12	0.387	0.118
13	0.621	0.423
14	0.487	0.294
15	0.334	0.097
16	0.562	0.365
C1	0.052	0.048
C2	0.032	0.034
C3	0.048	0.051
C4	0.043	0.048

Serial nos. 1–16: sera from *Curvularia* hypersensitive patients. Serial nos. C1–C4: sera of healthy individuals were considered as control. ELISA positivity was determined with reference to negative control. Sera showing OD ≥ 3 times the average value of control serum (0.059) was considered positive.

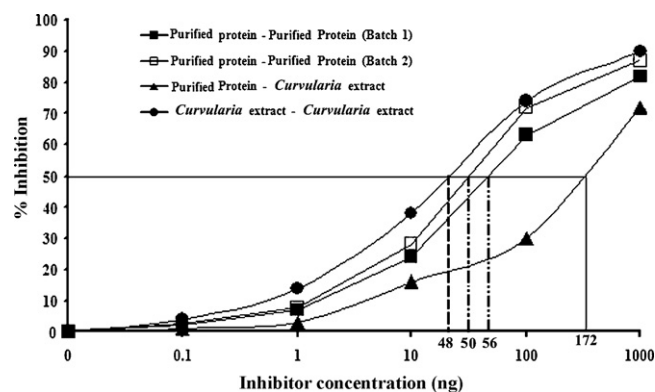


Fig. 4. ELISA inhibition. IgE inhibition of purified protein (3 batches) with self protein and *Curvularia* extract with *Curvularia* sensitive patients' pooled sera. The serum was incubated with 0.1–1000 ng of *Curvularia* extract or recombinant protein as inhibitor.

The allergenicity of purified protein was assessed by its ability to induce histamine release from sensitized basophils. Basophils sensitized with *Curvularia* positive patients' sera ($n = 16$) individually showed histamine release in the range of 25–53% on challenge with 5 ng of *Curvularia* extract (protein). On challenge with purified

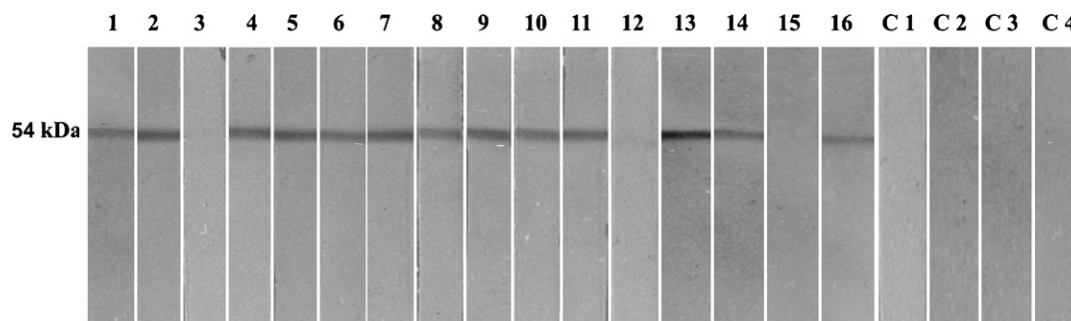


Fig. 3. IgE binding with individual patients' and control sera. IgE binding of recombinant purified 54-kDa protein with individual patients' (1–16) sera on immunoblot; C1–C4, IgE binding with control sera. Purified protein recognized 13/16 of *Curvularia* sensitive patients' sera whereas controls did not show binding with protein.

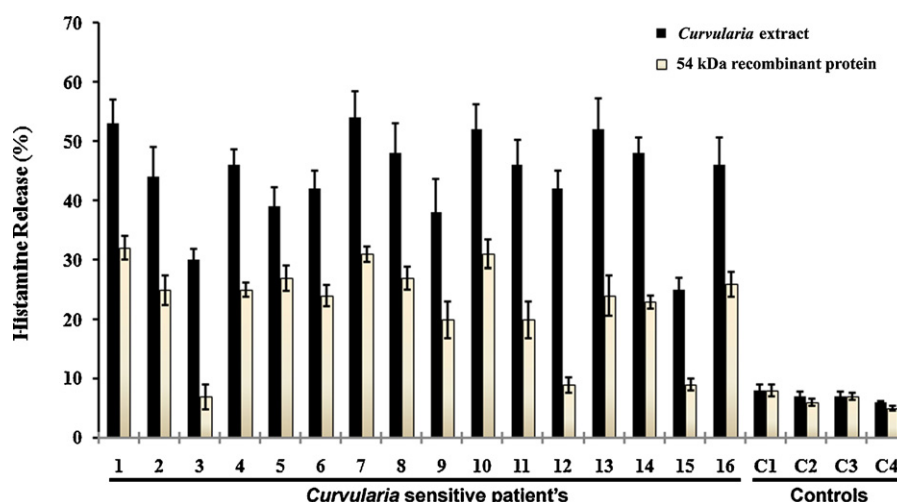


Fig. 5. Histamine release assay. For histamine release assay, basophils in leukocyte suspension were stripped off the bound IgE. The basophils were sensitized with sera of *Curvularia*-sensitive patients. The sensitized basophils were stimulated with *Curvularia* extract or 54-kDa purified protein. The histamine released in cell supernatant was quantified and expressed as percentage of total histamine content.

54-kDa protein (5 ng), 13/16 of patients' samples showed significant histamine release in the range of 20–32%. Patient samples 3, 12 and 15 with low IgE, released < 10% of histamine on stimulation with 54-kDa protein. Basophils sensitized with control sera showed 5–9% histamine release in response to these antigens (Fig. 5). Here, histamine release of >10% was taken as cut-off to grade the positive results.

Discussion

Fungal extracts prepared from spores and mycelia are variable in allergen composition and content (Tripathi et al., 2006). The commercially available natural extracts also show variation from one manufacturer to another, may be due to source material of different batches and location or method of preparation (Esch, 1997). Recombinant proteins are homogenous preparations with defined allergenic potency that show promise for component resolved diagnosis and therapy of allergic diseases (Wiedermann, 2005). In the present study, a 54-kDa subtilisin like serine protease of *C. lunata* was identified by immunoscreening of *C. lunata* cDNA library. It was cloned, expressed and characterized by bioinformatics and immunobiochemical methods.

Sequence similarity search against Swissprot database (<http://expasy.org/sprot/>) revealed significant homology to subtilisin like serine protease from *P. tritici* (94%), *C. cladosporoides* (89%), *A. fumigatus* (85%), *P. oxalicum* (84%), *P. citrinum* (83%), *P. chrysogenum* (80%) and *C. albicans* (77%). Further, it showed the presence of conserved serine protease motifs of subtilase family at position 215–225 and 379–389 indicating the protein to be a subtilisin like serine protease. The protein showed homology with fungal protease allergens in SDAP database. The protein sequence showed a hydrophobic stretch MKYSLIAALPALAAA, similar to the N-terminal leader sequence of Pen ch 18 and Pen o 18 allergen (Shen et al., 2001, 2003). Sequence homology with other serine proteases also shows the presence of a prosequence.

The protein was expressed in *E. Coli* and it separated at 54 kDa on SDS-PAGE. On the basis of sequence homology, conserved domain and its reactivity with anti-Cur I 1 (alkaline serine protease), the 54-kDa protein was detected to be a serine protease. However, recombinant serine protease lacked proteolytic activity. Most of the subtilisin like serine proteases are produced as precursor molecules where signal peptide helps in membrane translocation whereas prosequence functions as self-inhibitor (Pöll et al., 2009). It will be

a challenge to activate Cur I 4 to have enzymatic activity by removing the prosequence. Serine proteases from diverse sources such as fungi, mites and cockroach were recognized as major allergens associated with IgE mediated reactions (King et al., 1996; Chou et al., 2002; Gupta et al., 2004; Sudha et al., 2008). House dust mite serine proteases of group 3 (trypsin), group 6 (chymotrypsin) and group 9 (collagenase) allergens showed IgE binding in the range of 92–100% (King et al., 1996). A recent study indicates significant IgE cross-reactivity between subtilisin like serine proteases namely Cla h 9, Asp f 18 and Pen ch 13 (Pöll et al., 2009). In the present study, recombinant 54 kDa protein demonstrated IgE binding with >80% of *C. lunata* sensitive patients' sera by ELISA and immunoblot indicating it to be a major allergen. Three patients (nos. –3, 12, 15) showing low IgE binding with 54 kDa protein may be hypersensitive to other *C. lunata* protein(s). Previously, purified allergens of *C. lunata* (Cur I 1, Cur I 2 and Cur I 3) demonstrated IgE binding in the range of 67–80% of *Curvularia* allergic patients' sera (Gupta et al., 2004; Sharma et al., 2006, 2008).

The potency of allergen extracts from molds namely, *A. alternata*, *C. albicans*, *E. purpurascens* and *P. chrysogenum* was determined by ELISA inhibition and/or histamine release assay (Akiyama et al., 1996; Chou et al., 2002; Shankar et al., 2006; Kukreja et al., 2009). Studies show that fungal extracts required 40–100 ng of self protein for 50% inhibition of IgE binding in ELISA (Bisht et al., 2002; Kukreja et al., 2009) whereas fungal allergens showed EC₅₀ values in the range of 1.1–2.7 ng (Shankar et al., 2006; Sharma et al., 2008). In the present study, purified 54 kDa protein showed dose dependent inhibition in competitive ELISA using same protein as inhibitor. The protein seems highly potent and it required 50–56 ng of same protein for 50% inhibition of IgE binding (EC₅₀) on solid-phase coated 54 kDa protein. The challenge with fungal allergens such as Cur I 3, Pen ch 13 and 12 kDa allergen from *E. purpurascens* released histamine in the range of 12–73% (Chou et al., 2002; Sharma et al., 2008; Kukreja et al., 2009). The challenge with purified 54 kDa protein induced 20–32% histamine release in 13/16 of patients' samples. The patients with low specific IgE titer against 54-kDa protein demonstrated <10% histamine release.

An imbalance in the level of proteases and antiproteases results in a defective lung epithelial barrier thus providing access to allergens (Smith and Harper, 2006). Previous studies indicate that serine protease activity in whole body cockroach extract may be responsible for inducing proinflammatory effects on human bronchial epithelial cells by stimulation of protease-activated receptor-2

(PAR-2), leading to progression of airway inflammatory diseases (Sun et al., 2001). Proteolytic activity facilitates entry of other allergens into the epithelium and plays a role in allergen presentation (Sudha et al., 2009). Serine proteases are cross-reactive allergens and are present in many extracts (Shen et al., 2001, 2003; Pöhl et al., 2009). Cur l 4 with no serine protease activity is a good candidate for immunotherapy and will inhibit its presentation as well as presentation of other allergens. The absence of proteolytic activity may be due to improper folding of the protein. The conformational B-cell epitopes have been shown to be strongly reduced in unfolded recombinant allergens (Dudler et al., 1992; Müller et al., 1995) and as a consequence inhibits mediator release from effector cells. All relevant T-cell epitopes of the allergen are preserved to interact with cells for the induction of protective immunity.

In conclusion, a 54 kDa major allergen of *C. lunata* was cloned, expressed, characterized and showed biological activity. The molecule can be further explored for cross-reactivity among fungi. The availability of such cross-reactive recombinant molecules may serve as a candidate for diagnosis and therapy of multiple fungal sensitivity.

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