

Cloning, Recombinant Expression and Activity Studies of a Major Allergen “Enolase” from the Fungus *Curvularia Lunata*

VIDHU SHARMA,^{1,3} RATNA GUPTA,¹ ANUPAM JHINGRAN,^{1,3} BHANU PRATAP SINGH,¹
SUSHEELA SRIDHARA,¹ SHAILENDRA NATH GAUR,² and NAVEEN ARORA^{1,4}

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Recombinant allergens are required to study allergy at the molecular level and are helpful tools for the improvement of diagnosis and therapy. In the present study, enolase was expressed from *Curvularia lunata* and analyzed for its immunological reactivity as an allergen. cDNA library was synthesized in λ zap vector and screened with sera obtained from *C. lunata* allergic patients. A cDNA clone with an ORF of 1.3 kb showed homology to enolases from different fungal sources. It was expressed in *E. coli*, purified from inclusion bodies yielding 0.5 mg/L and showed enzyme activity of 48 units/mg. It resolved as 48-kDa band on SDS-PAGE and was recognized by all the individual *Curvularia* positive patient sera in immunoblot and ELISA. rCur l 2 stimulated patients' PBMCs and supernatant of these cells showed elevated levels of Th 2 cytokines. Ten B cell epitopes were predicted using computational software and one showed 90% homology to an important IgE epitope of Cla h 6. The various parameters predicted by computational approach can be validated later as a future study to draw conclusive evidence about putative antigenic epitopes. This can further help in generating knowledge about residues important for IgE binding and developing therapeutic modalities.

KEY WORDS: Enolase; rCur l 2; *Curvularia*; recombinant; epitope.

INTRODUCTION

Type I allergy is an IgE mediated hypersensitivity induced by exposure to allergen affecting 25% of the world's population (1). Many environmental agents are respon-

sible for such altered immune reactions, especially pollens, fungal spores, dust mite, animal danders, drugs, or even food. Amongst fungi, the genus *Curvularia* is an important inhalant allergen known to cause respiratory allergy disorders such as asthma and rhinitis (2). There are few reports from America (3) and Asian countries (4) demonstrating 18–28% prevalence of *Curvularia* sensitization. The characterization of *Curvularia* allergens becomes all the more important in this respect. Clinical studies carried out with *Curvularia lunata* extract in India exhibited markedly positive skin reactions (2+ to 4+) in 7–16% of patients having nasobronchial symptoms (5, 6). *C. lunata* has also shown cross-reactivity with different species of *Curvularia* and other prevalent fungi such as *A. alternata*, *A. fumigatus*, *C. herbarum*, *E. nigrum*, *F. solani*, etc (7, 8). Proteins of 12, 26, 31, 45, and 50 kDa from *C. lunata* were cross-reactive with these fungi (8). In an earlier study, a 45-kDa protein was demonstrated as a major cross-reactive allergen in *F. solani* and other fungi (9). Serine protease was earlier shown as an important allergen from *Curvularia* (10). Identification of more allergenic components will add to the knowledge of allergic manifestations caused by *Curvularia* considering the fact it is an important allergy causing fungi.

Many enzymes like proteases, transferases have been reported as important allergens from various sources (10, 11). Enolase, 45–48 kDa protein, has been observed as highly conserved fungal allergen (12). It is a glycolytic enzyme showing allergic potential in mold and latex allergic individuals. It has been studied from *A. alternata* (Alt a 6) and *C. herbarum* (Cla h 6) showing high IgE binding activity (13). Hev b 9, enolase from latex has also been cloned and its cross-reactivity with mold

¹Institute of Genomics and Integrative Biology, Delhi, India.

²Vallabhbhai Patel Chest Institute, Delhi, India.

³Dr. B.R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi, India.

⁴To whom correspondence should be addressed to Allergy and Immunology Section, Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road (near Jubilee hall), Delhi 110007, India; e-mail: naveen@igib.res.in.

Abbreviations used: CL: *Curvularia lunata*; IEF: Isoelectric focusing; PEP: Phosphoenol pyruvate; PGA: Phosphoglyceric acid; rCur l 2: recombinant Cur l 2.

enolases was studied (14). These signify the importance of obtaining enolase in recombinant form for further cross-reactivity studies and dissecting its epitope complexities at molecular level. The present study was aimed to clone and express enolase allergen from *C. lunata* followed by assessing its biological activity and analyzing its relevance as an allergen among *Curvularia* sensitive patients by immunological and biochemical methods.

METHODS

Total RNA and mRNA Isolation

Total RNA was isolated from 4-day-old *C. lunata* culture by mono-phasic solution of phenol and guanidium isothiocyanate using TRIzol reagent (Life Technologies). From total RNA, 7 µg mRNA were obtained and cDNA library was synthesized using 5 µg mRNA.

Construction of cDNA Library

The cDNA fractions (containing inserts, 0.5–2 kb) were pooled and 100 ng was ligated to Uni-ZAP XR Vector at 12°C overnight. The ligation mix (1 µL) was packaged as per manufacturer's instructions.

Patient's Sera/Blood

The sera were collected from 20 patients suffering with allergic bronchial asthma, rhinitis, or both as per the case histories at out patient department in V.P. Chest Institute, Delhi. The diagnosis of asthma was ascertained as per guidelines of the American thoracic society and rhinitis was assigned to cases having any two of the symptoms such as sneezing, rhinorrhea, nasal blockage, postnasal drip, etc. for the last 2 years.

These patients showed marked positive reactivity (2 + to 4 +) to *C. lunata* extracts by intradermal tests as described earlier (15). The sera samples were pooled and adsorbed against *E. coli* XL-1 blue lysate (1 mg/mL) and used for screening of the cDNA library. The individual sera from these patients ($n = 20$) were used for ELISA. The sera with specific IgE $3 \times$ or more of the negative control were selected for immunoblot ($n = 15$). For lymphoproliferation assay, PBMCs were isolated using 10 mL blood from all the 20 patients. Blood from five healthy (nonatopic) individuals was collected as control samples. Institutional ethics committee approved the protocol and informed consent of each patient was obtained for blood collection and skin tests.

Screening of cDNA Library with Pooled Patient's Sera

Screening was performed using preadsorbed patient's serum at a dilution of 1:10 v/v in TBS. Briefly, the phages containing cDNA library were used to infect *E. coli* XL-1 Blue cells, plated on 90 mm NZY agar plate (density 300–400 plaques/plate) and incubated at 42°C for 4 h. The nitrocellulose disc soaked in 10 mM IPTG 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, 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.

DNA Sequencing

The phagemid DNA were amplified by PCR with T3/T7 primers. After an initial denaturation step at 95°C for 3 min, 1.5 U Taq polymerase was added and the DNA was amplified for 25 cycles on a thermal cycler (Perkin–Elmer Corp.) following denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min. The amplified product was purified using a DNA isolation kit (Biological Industries). To get the full length sequence of this cDNA, primer walking method using 2 pmol of A1 and A2 primers (forward primers); Primer A1: 5' GAG AGA TGC GTG TGT AGA GGC TGA A 3' and Primer A2: 5'GAG CTG CAG TTC CTG GTC AGA 3'. The oligonucleotides were synthesized on Gene Assembler Plus (Pharmacia-LKB). Automated sequencing was performed by Sanger's method on ABI-377, DNA Sequencer, Applied Biosystems.

Computational Analysis of Sequence and Homology Search

The ORF and deduced protein obtained from the nucleotide sequence were analyzed using DNASTAR software. The molecular weight, pI, secondary structure, antigenic stretches, net charge on the protein, etc. were determined using same software. The homology search was carried out using CLUSTAL W and Basic Local Alignment Search Tool (BLAST) algorithm available at National Center for Biotechnology Information.

Sub-Cloning in pET22b + Expression Vector

The sequenced cDNA fragment was ligated into pET22b + expression vector (Novagen). The primers A3:

5' G AAT TCG AAT TCA ATG GCC ATC ACA AAG ATC CAC 3' and A4: 5' CTC GAG CTC GAG CAA GTA GAG GTT AAT GGC GGT 3' were used to amplify cDNA fragment and had *Eco R I* and *Xho I*, respectively at flanking ends. The template was GC rich, thus different concentrations of $MgCl_2$ (1.5–4.0 mM), DMSO (5–10%), and different temperature gradients (annealing temp. from 48–69°C) were tried. Finally, the template was amplified using the reaction mix constituting 40 nM template, 1 × PCR buffer, 20 pmol each primer, 10% DMSO, 2.0 mM $MgCl_2$, and 1 mM dNTPs mix. The reaction mix was heated to 95°C for 10 min in a thermal cycler (Applied Biosystems) and 1.5U Taq polymerase was added for a two-step PCR reaction (i.e., melting at 94°C, 1 min and annealing and extension at 68.4°C, 3 min) for 30 cycles with a final extension at 72°C for 10 min. The PCR product was purified using Qiagen kit. The PCR product and pET22b+ were digested with *Eco R I* and *Xho I* restriction enzymes and ligated using T4 DNA ligase at 16°C overnight. The ligation mix was transformed into *E. coli DH5α* cells and selected on LB ampicillin plates. The positive clones obtained were confirmed by restriction digestion and PCR amplification. The plasmid containing this cDNA fragment was designated as pCL1.

Expression and Purification of Recombinant Allergen

The plasmid pCL1 was transformed into *E. coli BL21* cells, grown till O.D. 0.8, and induced with 1 mM IPTG for 4 h and harvested. The cell pellet was suspended in 50 mM Tris-HCl, pH-7.5, sonicated, and centrifuged at 15000 rpm for 30 min at 4°C. The expressed enolase was 10–15% of the total protein and was five fold more in the pellet as compared to the supernatant indicating the formation of inclusion bodies. It was confirmed by Western blot using rabbit sera raised against *Curvularia* extract (data not shown). To solubilize the inclusion bodies, pellet obtained after sonication was suspended in a mix containing 1.5% *N*-lauryl sarcosine, 1% Triethanolamine with 1 mM PMSF, and incubated with gentle rocking at 4°C overnight. It was centrifuged and supernatant was loaded onto Ni-NTA resin equilibrated with binding buffer containing 10 mM Tris.Cl, 100 mM NaH_2PO_4 , 0.5 M NaCl, pH 8.0 for binding. The his-tagged protein was eluted using an imidazole gradient (100–500 mM) in the binding buffer. The elution was maximum in 250 mM imidazole. The eluted fractions were dialyzed in 20 mM phosphate buffer to regain the appropriate folding of protein. Protein concentration was estimated using Lowry's method (16) and was designated as Cur l 2. (Submitted to WHO/IUIS Allergen Nomenclature Sub committee).

Enzyme-Activity Assay

The enolase activity was tested in the eluted fractions according to the method described earlier (17). Briefly, 1 mL of enolase substrate solution (50 mM Tris.Cl, pH 7.4, 2.7 mM magnesium acetate, 1.0 mM EDTA, 1.2 mM PGA) was taken in quartz cuvette. Different concentrations of the eluted protein were added to it and the change of absorbance recorded at 1 min interval at 240 nm.

Assessment of Allergenic Activity of rCur l 2

IgE binding of rCur l 2 was determined by ELISA and immunoblot using *Curvularia* hypersensitive patients' sera.

ELISA

Specific IgE ELISA was performed to determine allergenic activity of the recombinant protein using allergic patients' sera (18). Briefly, 100 ng of purified protein per well was coated in 100 μ L 50 mM carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed with PBS and blocked with 3% defatted milk at 37°C for 2 h, and incubated overnight with 100 μ L each of 20 individual fungal allergic patient's sera (1:10 v/v) diluted in defatted milk with PBS. Normal human sera were also tested as negative control ($n=5$). The plates were washed with PBS containing 0.05% Tween 20, incubated for 3 h at 37°C with antihuman IgE peroxidase labeled (1:1000 v/v, Sigma). After washing, color was developed with *o*-phenylene diamine and absorbance was read at 490 nm in an ELISA reader.

Immunoblot

Briefly, purified recombinant protein separated on 12% SDS-PAGE (19) was transferred to a nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 25% methanol at 150 mA for 3 h (20). The membrane was cut into strips, blocked, washed with PBS containing 0.05% Tween 20, and incubated overnight at 4°C with 15 hypersensitive individual patient's sera (1:10 v/v). The strips were again washed, incubated with antihuman IgE peroxidase (1:1000 v/v, Sigma) for 3 h at 37°C, and developed with diaminobenzidine in 0.08 M acetate buffer (pH 5.0).

Lymphocyte Proliferation on Antigen Challenge

PBMCs isolated from heparinized *C. lunata* hypersensitive patients' blood ($n=20$) by Ficoll hypaque centrifugation (Sigma Chemical Co.) were washed three times with PBS and resuspended in RPMI-1640 containing 25 mM HEPES, nonessential amino acids, B group

vitamins (in trace amounts), 23 mM sodium bicarbonate, 100 $\mu\text{g/mL}$ streptomycin, 100 U/mL penicillin (Sigma), and 10% fetal bovine serum. For lymphoproliferation assays, 10^5 cells/well were cultured in RPMI media and challenged with 5 μg each of *Curvularia* extract and Cur 1 2. Phytohemagglutinin (5 μg) was used as positive control and no stimulant was added in negative control. The culture plates were incubated for 72 h at 37°C and supernatant collected for cytokine assay. Cells were washed with RPMI and incubated for 2 h with 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 100 $\mu\text{g/mL}$). The cells were lysed in acidic isopropanol solution and the absorbance read at 570 nm (15).

Cytokine Analysis in Culture Supernatant

IL-4, IL-5, and IFN- γ were estimated using ELISA kits following manufacturer's protocol (BD Pharmingen OptEIA kits). Briefly, capture antibody (1:250 dil.) for each cytokine was coated separately on microtitre plates in carbonate buffer, pH 9.6 and incubated overnight at 4°C. The wells were blocked with PBS containing 10% fetal bovine serum at 37°C for 1 h. After washing, standards (5–500 ng), 100 μL of 1:2 diluted sample or controls were added to wells and incubated at 37°C for 3 h. The plates were washed again, incubated with biotinylated detector antibody labeled with avidin peroxidase at 37°C for 1 h. The color was developed using *o*-phenylene diamine substrate. The reaction was stopped with 2 N H_2SO_4 and read at 570 nm (15).

Statistical Analysis

Parametric statistical analysis was performed to compare the means of specific IgE values and lymphoproliferation indices between healthy controls and allergic individual's observations using Student's *t* test. The comparison of means in various parameters was analyzed by one-way ANOVA [Analysis of Variance] (Graphpad software). The $p < 0.05$ was considered significant in all cases. All the results are represented as bar graphs with mean $\pm$ SD. The box plots of medians with interquartile range were made to analyze the comparisons of calculated parameters.

RESULTS

cDNA Library Screening and Sequencing of pCL 1

The titer of cDNA library was 1×10^9 . Twelve clones were identified after screening the library with pooled sera

obtained from *C. lunata* allergic patients. cDNA clone of approximately 1500 bp sequenced by primer walking method and pET vector containing cDNA fragment was designated pCL1. Its ORF was 1323 bp (ACCESSION No. AY034826, Fig. 1a) encoding a mature protein of 441 amino acids.

Computational Analysis

Comparison of translated coding sequence of cDNA fragment with NCBI database showed homology with enolase from various sources at both DNA and protein level. The sequence alignment of Cur 1 2 with enolase from other sources is given in Fig. 1b. At protein level, it was similar to *A. alternata* (88%) [Q9HDT3], *Candida albicans* (71%) [P30575], *C. herbarum*, (84%) [P42040], *Penicillium citrinum* (79%) [AAK51201], *Hevea brasiliensis* (70%) [Q9LEI9], and human (60%) non-neuronal α enolase [P06733]. Its molecular weight was 47.8 kDa with pI of 5.57 demonstrating it to be an anionic protein. The sequence of rCur 1 2 showed the presence of conserved motif SHRSGETED (14) Fig. 1a. The motif search by Gene Runner software located DDLTVTNP as enolase motif. The crucial residues important for enzyme activity in *S. cerevisiae* have been identified as E211, K345, and H373 while Cur 1 2 has E211, K347, and H375 (Fig. 1a). Similarly, the amino acid residues required for metal binding in *S. cerevisiae* are S39, D246, E 295, and D 320 which are also comparable to Cur 1 2 i.e., S40, D 246, E295, and D322 boxed in Fig. 1a (14). The enolase motif conserved in other fungal enolases is depicted in the sequence alignment of *C. lunata* sequence (Fig. 1b). Further, antibody binding regions i.e., epitopes of enolase protein were predicted based on computational algorithms considering the parameters such as secondary structure, surface probability, hydrophilicity, flexibility, etc. Ten major antigenic regions were predicted as presented in Fig. 1c based on Jameson Wolf antigen prediction algorithm. The antigenic region 138–162 in rCur 1 2 was 90% homologous and 85% identical to an important epitope from *C. herbarum* enolase i.e., Cla h 6 (13).

Cur 1 2 Amplification, Expression, and Purification

rCur 1 2 has a high GC content and was therefore amplified by using 10% DMSO in 2-step PCR cycle. The amplified fragment was approximately 1.3 kb. Figure 2 (lane 1 and 2) shows the uninduced and induced cell culture lysate, respectively. Figure 2, lane 3 shows the purified rCur 1 2 band at 48-kDa yielding 0.5 mg-purified protein/L of culture.

Enzyme Activity of rCur 1 2

The specific enzyme activity of rCur 1 2 was 48 units/mg of protein. The standard enolase from yeast (Sigma) showed the activity of 50 units/mg. This shows that rCur 1 2 is biologically active. Sarcosyl used to solubilize inclusion bodies for purification tends to denature the protein. In the present study, dialysis against low strength phosphate buffer was able to renature rCur 1 2 and thus showed enzymatic activity, which was comparable to commercial yeast enolase.

Allergenic Activity of rCur 1 2

The IgE binding activity of rCur 1 2 was investigated by testing individual sera of *C. lunata* allergic patients in immunoblot and ELISA experiments. For ELISA, all the 20 patients' sera were tested whereas for immunoblot patients having specific IgE three times or more of the negative control were probed. In ELISA, patients 3, 6, 10, 15 showed slightly higher specific IgE against Cur 1 2 than *Curvularia* extract. Probably these patients are specifically sensitive to Cur 1 2 and therefore show higher

specific IgE with purified protein than native extract. In immunoblot, all 15 patients reacted with rCur 1 2 demonstrating it as a major allergen (Fig. 3, Lane 1–15). Serum pool from nonatopic healthy individuals and pollen-allergic patients were taken as controls showing no binding on immunoblot.

ELISA with these samples ($n = 15$) showed three fold or more IgE binding than controls. The mean value for specific IgE against *Curvularia* extract is 0.47 ± 0.221 and for Cur 1 2 is 0.42 ± 0.21 compared to the normal controls with mean value 0.078 ± 0.05 (Fig. 4). These results show that rCur 1 2 is a major allergen of *C. lunata* with immunological reactivity comparable to whole extract.

Cytokine Analysis and Lymphocyte Proliferation on rCur 1 2 Challenge

rCur 1 2 stimulated proliferation in the PBMCs from *Curvularia* sensitive patients. The range of stimulation indices for various samples and controls is presented in Fig. 5. There was three to fivefold stimulation

5'	11	21	31	41	51	61	71	81	91						
1 M A I T K I H A R S U Y D S R G N P T U E U D I U T E T G L H R A	1 A T G G C C A T C A A A G A T C C C C G C T C T G T C A C G A C T C T C G C G T A A C C C C A C C G T C G A G G T T G A C A T T G T C A C C G A G A C T G G C C T C C A C C G T G C C A	34 I U P S G A S T G S H E A C E L R D G D K T K W G G K G U T K A U A	101 T T G T C C C T C T G G T G C C T C C A C C G G T C C C H C A G G G C T G C G A A C T C C C G G A T G T G T G T G A C A K T A C C G A A G T G G G T G C C A A G G T G T C A C C A G U T K A U A A G G T G T C C A	68 N U N D I I A P A L I K E K L D U K D Q S A U D A F L N K L D G T	201 C A A C G T C A A C G A C T A T T G C C C T G C T C T C A T C A A G G A G A A G C T C G A C G T C A A G G A C C A G T C T C C G G T A G A T G C T T T C C T C A A C A A G C T T G A T G G C A C A	101 E N K T K L G A N A I L G U S H A I A K A A A E K G A E K G U P L Y A H	301 G A A A C A A G A C C A A G T C G G T G C C A A T T G C C A A A G C C G C G T G C C G A A A G G G T G T C C C T C T C T A C G C A H A T A C G C A C A C A	134 I S D L A G T K K P Y U F R F L S K N U L N G G S H A G G F L L F Q	401 T C T C C G A C T T G G T G G C A C C A A G A G C C T T A C G A A C G T C C C C A C G C C G G T G G T C T C T C C C A C G C C G G T G G T T C T T G C T T T T C C A	168 E F H I A P A K T F A E A L R I R Q G A E U V Q K L K A L T K K T	501 A G A G T T A T G A T C G C C C C C G C C A A G A C C T T C G C C G A G G C C C T C C C G A T T C A G A G G T C T A C C A G A A C T C A G A C T C A C A G C C T C A C A A G A A C C	201 Y G Q S A G N Y G D E G G U A P D I Q T A E E A L D L I U D A I E	601 T A C G G T C A G T C T C C C G T A A C T A C G G T G A C F A G G G T G G T G C G C C C C G A C A T T G C G A C G C C A T T A G A G C C A I E G C C A T T A G A G	234 A A G H T G Q I K I A M D U A S S E F F K D D E K K Y D L D F K N P	701 C C G C C G T C A C A C C G G T C A G A T T A A G A T T G C C A T G A C C C C T C T C T C A G A G A T T C A C C A G A C C A G A G A C T C A C A G A A G A C T C A C A G A A G A C C

Fig. 1. (a). The nucleotide and translated protein sequence of rCur 1 2. The upper lane shows the amino acid sequence and second lane shows the nucleotide sequence. The conserved motif is in rectangular red box. The residues required for enzyme activity are in circles and metal-binding amino acid residues are in boxes. (b). The sequence alignment of rCur 1 2 with enolases from other fungi. The residues that match the consensus are represented as (-). (c). Computational prediction of rCur 1 2 antigenic stretches by DNASTAR software. It identifies the antibody binding sites by Jameson Wolf antigen prediction algorithm.

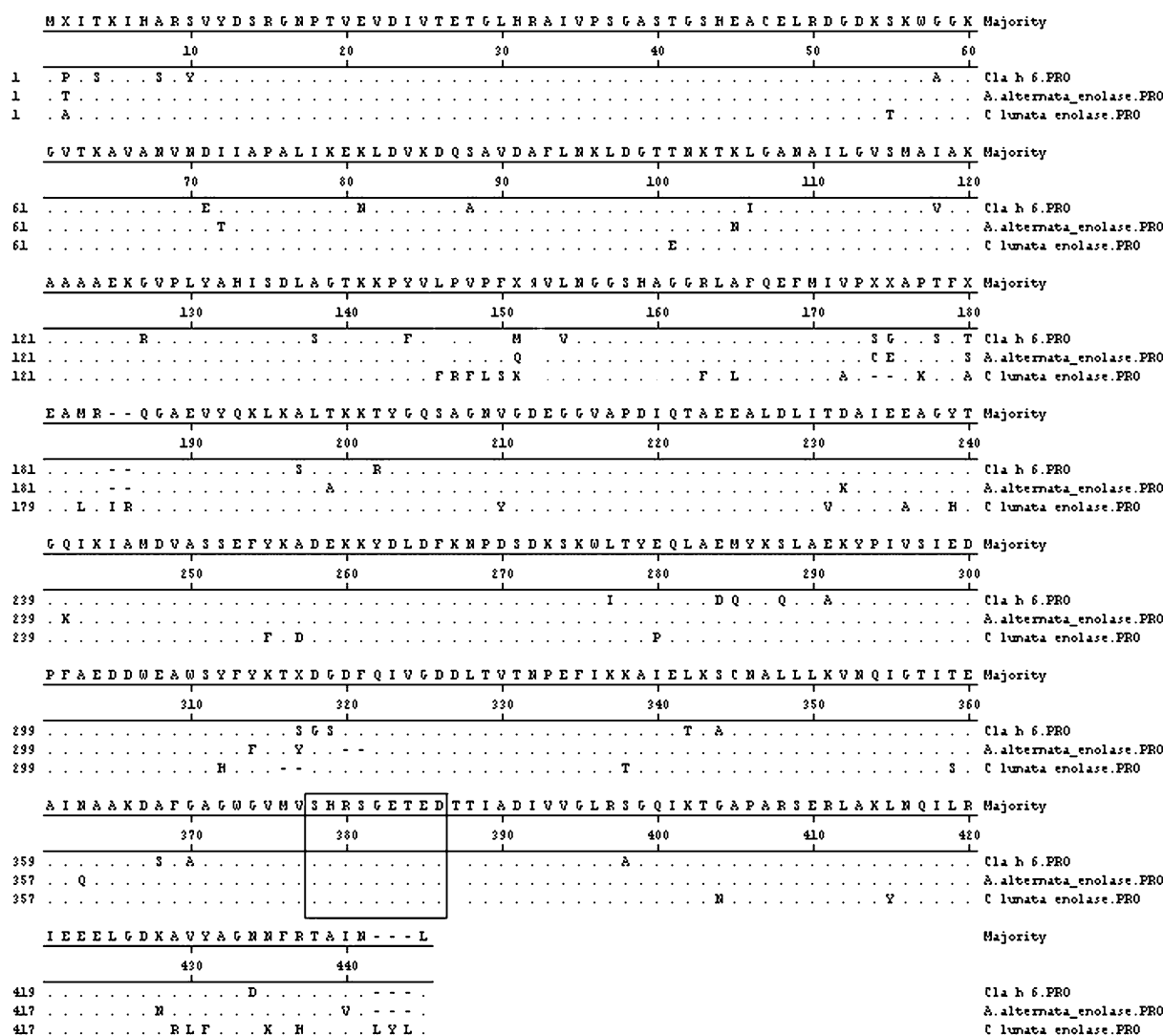


Fig. 1 b

observed in patient's PBMCs compared to the normal controls. Stimulation indices for *Curvularia* extract and rCur 1 2 were 0.532 ± 0.098 and 0.364 ± 0.18 , respectively whereas the healthy control values were below 0.1 i.e., 0.061 ± 0.032 . rCur 1 2 and *Curvularia* extract showed statistically significant correlation with respect to proliferation (P value = 0.88). Statistically significant difference between healthy controls and patients' ($p < 0.05$) was also observed.

IL-4, IL-5, and IFN- γ were estimated in the culture supernatants patient's PBMCs and healthy controls. It demonstrated high IL-4 and IL-5 levels for

both crude extract and rCur 1 2 as compared to healthy controls and exhibited significant difference ($p < 0.05$). IL-4 levels were 158 ± 31 pg/mL for purified protein and 171.6 ± 34 pg/mL for *Curvularia* extract (Fig. 6a), IL-5 levels were 140 ± 35 pg/mL for purified protein and 159 ± 42 pg/mL for *Curvularia* extract (Fig. 6b). IFN- γ levels (Th 1 cytokine) on stimulation with *Curvularia* extract (89 ± 23 pg/mL) and purified protein (59 ± 21 pg/mL) were comparable to healthy controls (Fig. 6c). The control samples taken here are the PBMCs isolated from healthy individual's blood not showing significant proliferation and cytokine secretion.

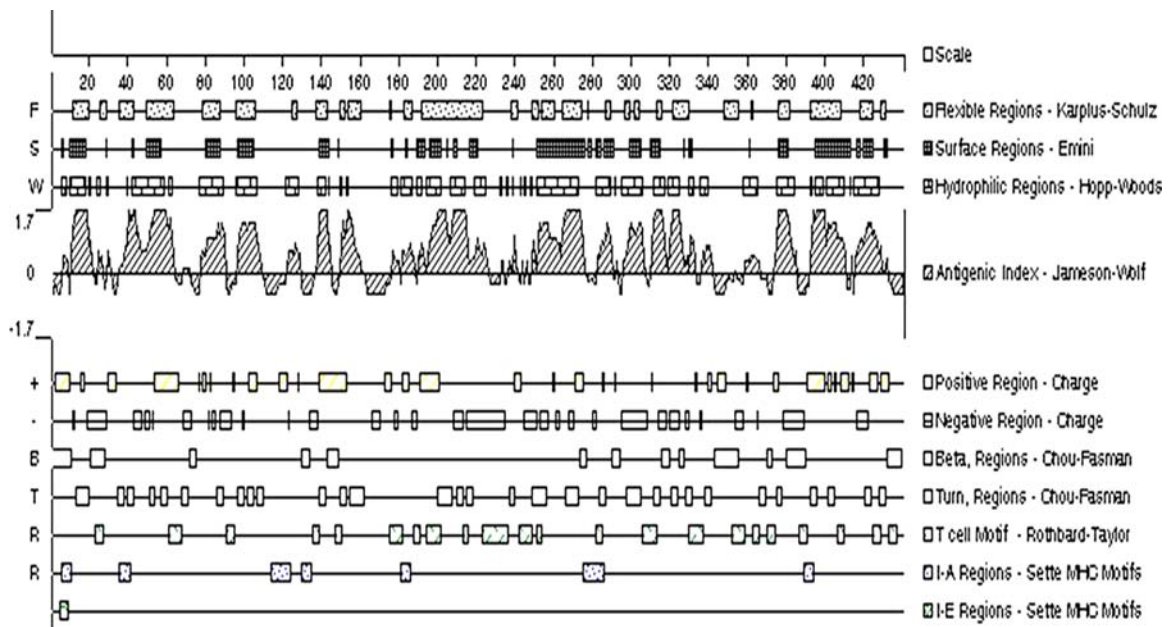


Fig. 1 c

DISCUSSION

Recombinant allergens have shown promise for specific diagnosis and therapy of allergy disorders (21). These pro-

teins offer new scope for molecular characterization of allergens and will aid in mapping the epitopic regions yielding valuable information on common antigenic/allergenic stretches of proteins. In the present study, a 48-kDa major allergen (enolase) of *C. lunata* was cloned, expressed, and characterized. Enolase was identified as an important allergen from various fungi, latex (12–14, 22–26). It has potential to be used for component-based diagnosis and therapy. The computational analysis of rCur l 2 showed significant homology with enolase at protein level from *A. alternata* (88%), *Candida albicans* (71%), *C. herbarum*, (84%), and *Penicillium citrinum* (79%), *Hevea brasiliensis* (70%), and human (60%) enolase.

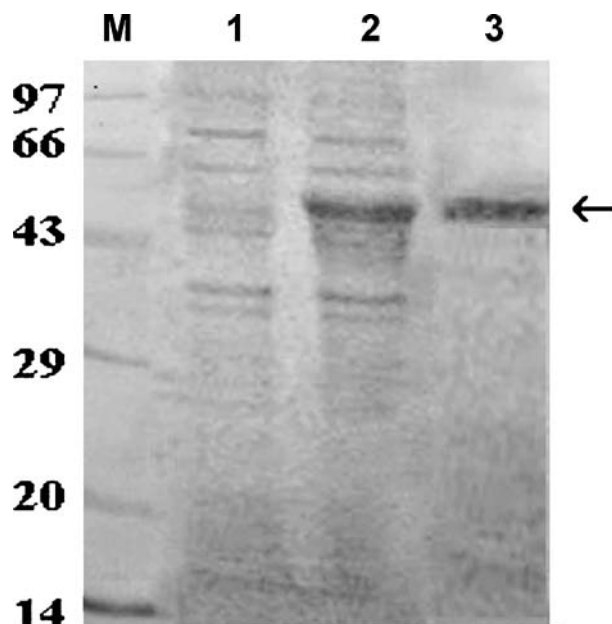


Fig. 2. SDS-PAGE profile showing the expression and purification of Cur l 2. Lanes 1 and 2: uninduced and induced cell culture lysate, respectively. Lane 3 shows the purified rCur l 2 indicated by an arrow. Lane M: Molecular weight marker.

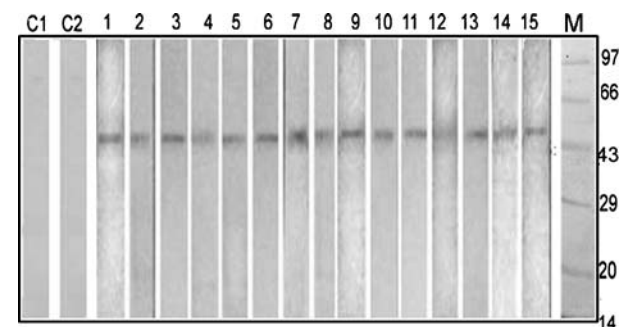


Fig. 3. IgE immunoblot of rCur l 2 using individual *Curvularia* positive patient sera. All the 15 patients recognized rCur l 2 showing it a major allergen. Lane C1: with serum pool of healthy individuals. Lane C2: with serum pool of pollen allergic sera. Lane M: Molecular weight marker.

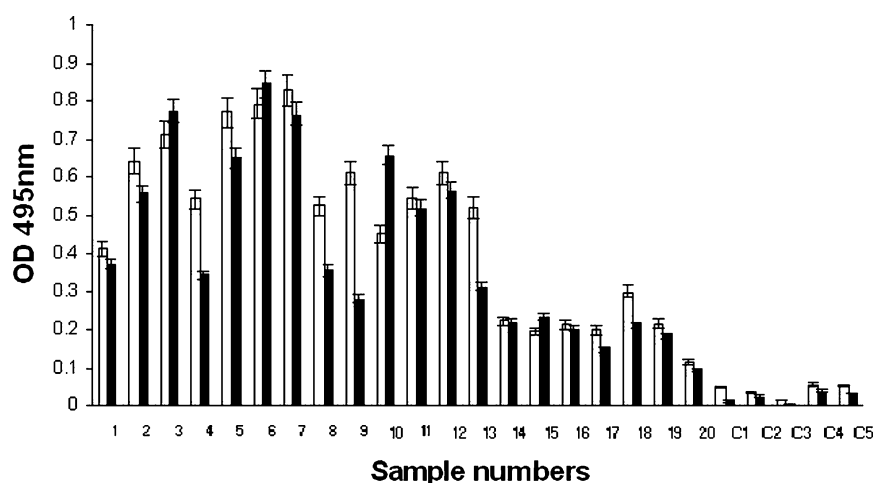


Fig. 4. IgE specific ELISA values against rCur 1 2 (■) and *Curvularia* extract (□) in 20 hypersensitive patients (Lanes 1–20). C1–C5 is healthy control samples. The mean $\pm$ SD for rCur 1 2 is 0.479 ± 0.221 and for *Curvularia* extract is 0.551 ± 0.195 . Blank value 0.098 ± 0.023 .

It showed the presence of conserved enolase motifs at position 374–382. The three-dimensional structure of *S. cerevisiae* showed the presence of two Mg^{2+} and S39, D246, E 295, and D 320 are considered important for metal binding (14). These correspond to S 40, D246, E295, and D322 in *C. lunata* enolase.

rCur 1 2, enolase from *C. lunata*, was identified as a major allergen recognized by all the hypersensitive patients' sera. It demonstrated high specific IgE antibodies ($3 \times$ or more) in *C. lunata* sensitized patients than nonatopic controls. Lymphocyte proliferation with rCur 1 2 in PBMCs

of patients' sample showed its high antigenic potential which was also seen for *Candida* enolase (26). The elevated levels of Th 2 cytokines in culture supernatants after stimulation with rCur 1 2 signifies the allergenic activity of this protein. *Curvularia* extract also showed high specific IgE values, lymphoproliferative activity and Th2 cytokine in *Curvularia* patients' PBMCs. The specific IgE value in few patients namely 3, 6, 10, and 15 was slightly higher than whole extract. This could be due to specific sensitization of patients to rCur 1 2. Enzymatic activity of rCur 1 2 was 48 units/mg and was comparable to the activity

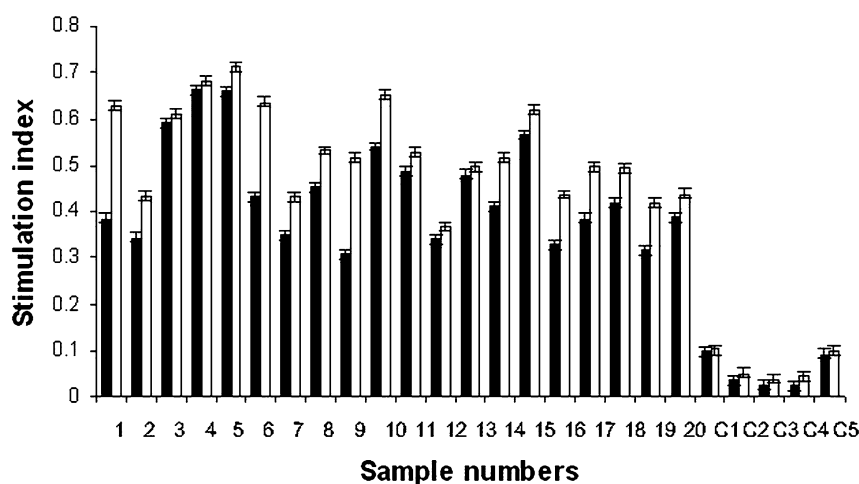


Fig. 5. Lymphoproliferation assay using *Curvularia* positive patient's PBMCs with rCur 1 2 (■) and crude *Curvularia* extract (□). C1–C5: healthy nonatopic control samples. The mean $\pm$ SD for rCur 1 2 is 0.364 ± 0.18 and that for crude extract is 0.532 ± 0.098 . Blank value without stimulant 0.112 ± 0.043 .

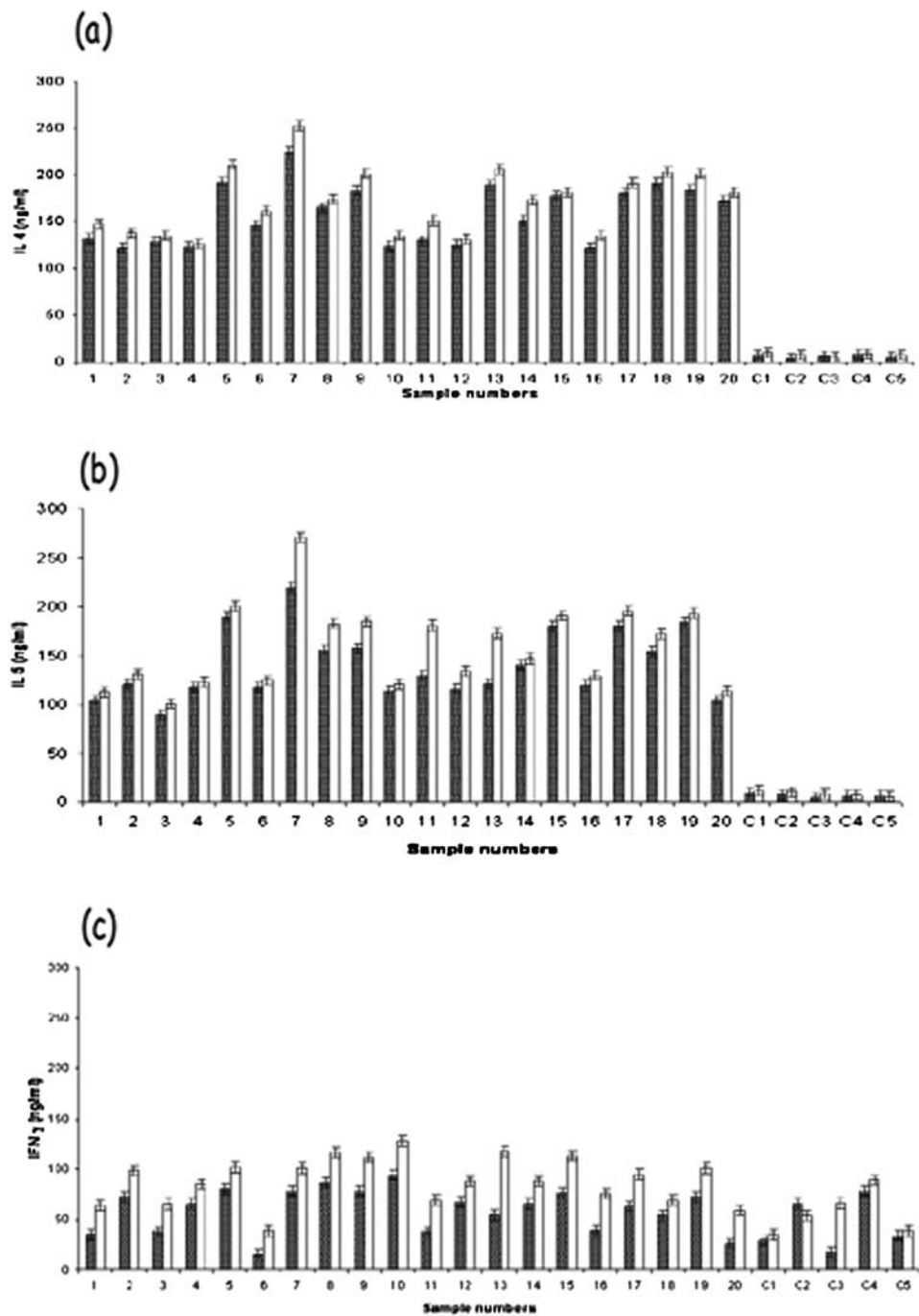


Fig. 6. Cytokine secretion in the culture supernatant of allergen stimulated PBMCs from patients (1–20) rCur 12 (■) and crude *Curvularia* extract (□). Healthy nonatopic controls (C1–C5). (a) IL-4 mean $\pm$ SD for rCur 12 is 158 ± 31 and for *Curvularia* extract is 171.6 ± 34 . (b) IL-5 mean $\pm$ SD for rCur 12 is 140 ± 35 and for *Curvularia* extract is 159 ± 42 . (c): IFN- γ mean $\pm$ SD for rCur 12 is 59 ± 21 and for *Curvularia* extract is 89 ± 23 .

of enolase from *Candida* which was 65–70 units/mg with PGA as substrate (17).

Peptide 9 (120–189) was shown to be IgE reactive by epitope mapping studies from *Cladosporium* enolase (13).

The molecular modeling of *C. herbarum* enolase showed that it is exposed to the solvent and may therefore facilitate interaction with IgE antibody. In the present study, computationally predicted epitopic stretches of rCur 12

demonstrated 10 major antigenic sites using Jameson-Wolf antigenicity prediction algorithm taking into account surface probability, hydrophilicity, flexibility, solvent accessibility, etc (27). The experimental validation of computationally predicted rCur 1 2 epitopes and investigation on cross-reactivity at the epitopic level are expected to give new diagnostic and therapeutic agents for fungal allergy.

In conclusion, rCur 1 2, a 48-kDa major allergen of *C. lunata* was cloned, expressed, and characterized as enolase. Further, rCur 1 2 demonstrated effector cell function in patients' PBMC and has potential for component based immunotherapy/diagnosis.

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