

Serine Protease Activity of Cur 1 1 from *Curvularia lunata* Augments Th2 Response in Mice

Prabhanshu Tripathi · Neetu Kukreja · B. P. Singh · Naveen Arora

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Abstract

Rationale Studies with mite allergens demonstrated that proteolytic activity augments allergic airway inflammation. This knowledge is limited to few enzyme allergens.

Objective The objective of this study is to investigate the effect of serine protease Cur 1 1 from *Curvularia lunata* in airway inflammation/hyper-responsiveness.

Methods Cur 1 1 was purified and inactivated using a serine protease inhibitor. Balb/c mice were sensitized with enzymatically active Cur 1 1 or *C. lunata* extract. Sensitized mice were given booster dose on day 14 with active or inactivated Cur 1 1. Intranasal challenge was given on day 28, 29, and 30. Airway hyper-responsiveness was measured by plethysmography. Blood, bronchoalveolar lavage fluid (BALF), spleen, and lungs from mice were analyzed for cellular infiltration, immunoglobulins, and cytokine levels. **Results** Mice challenged with enzymatically active Cur 1 1 demonstrated significantly higher airway inflammation than inactive Cur 1 1 group mice ($p < 0.01$). There was a significant difference in serum IgE and IgG1 levels among mice immunized with active Cur 1 1 and inactive Cur 1 1 ($p < 0.01$). IL-4 and IL-5 were higher in BALF and splenocyte culture supernatant of active Cur 1 1 than inactive Cur 1 1 mice. Lung histology revealed increased eosinophil infiltration, goblet cell hyperplasia and mucus secretion in active group.

Conclusion Proteolytic activity of Cur 1 1 plays an important role in airway inflammation and the inactivated Cur 1 1 has potential to be explored for immunotherapy.

Keywords Airway hyperactivity (AHR) · animal model · fungus · allergen · protease

Abbreviations

BAEE	α -benzoyl-L-arginine-ethyl ester hydrochloride
BALF	bronchoalveolar lavage fluid
BSA	bovine serum albumin
CBB	Coomassie brilliant blue
EDTA	ethylenediamine tetraacetic acid
EPO	eosinophil peroxidase
FCS	fetal calf serum
HE	hematoxylin–eosin
HTAB	hexadecyl trimethyl ammonium bromide
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
OD	optical density
OPD	<i>o</i> -phenylenediamine
OVA	ovalbumin
PAR	protease activated receptors
PAS	periodic acid-Schiff
PHA	phytohemagglutinin
PMSF	phenyl methyl sulphonyl fluoride
RPMI	Roswell Park Memorial Institute

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Introduction

Asthma is one of the most common chronic airways disease [1, 2]. It is characterized by airway inflammation that manifests airflow obstruction and hyper-responsiveness.

Sensitivity to airborne biogenic particles is considered a leading cause of allergic asthma. The inhalant allergens responsible for allergies have been isolated from several sources and characterized by immunologic, proteomic, molecular, and structural approaches [2–6]. The precise mechanism(s) employed by allergens in inducing atopic asthma is, however, unclear. Studies suggest that properties other than immunogenic (structural) presentation may contribute to allergenicity [7–9]. Proteolytic activity of allergenic molecules is one such property that endows molecules with ability to permeate through the epithelial defense barriers of host, thereby contributing to allergenicity [9].

Proteases from several sources e.g. *Dermatophagoides pteronyssinus* (Der p 1, Der p 3 and Der p 9), *Periplaneta americana* (Per a 10), *Aspergillus fumigatus* (Asp f 13), *Epicoecum purpurascens* (Epi p 1), *Penicillium chrysogenum* (Pen ch 13), *P. citrinum* (Pen c 1), and *P. oxalicum* (Pen o 18), etc. have been reported as important allergens having proteolytic activity [10–18]. Der p 1, a cysteine protease allergen, from dust mite *D. pteronyssinus* has been shown to subvert the regulatory processes controlling IgE production thereby favoring pro-allergic responses. Fungal proteases have been implicated in facilitating immune response by promoting release of pro-inflammatory cytokines. Despite a large body of evidence suggesting the role of hydrolytic activity of proteases in inducing inflammation, the actual effect of protease induced inflammation on induction of asthma symptoms like airway hyper-responsiveness has not been explored.

Curvularia lunata is among the predominant airborne molds inducing sensitization in many (7–16%) atopic individuals the world over leading to ailments like allergic rhinitis and asthma [19]. Immunoblotting with *C. lunata* positive patients' sera demonstrated 5–12 IgE reactive proteins of 12, 31, 45, and 53 kDa are shared among different *Curvularia* species [20]. Gupta et al. (2004) isolated a 31 kDa allergen from *C. lunata* spore mycelial extract having N-terminus sequence showing 92% identity with *A. oryzae*, *A. fumigatus* and *A. flavus* alkaline serine protease precursor. The allergen showed protease activity on different substrates and was designated as Cur l 1 [21]. The present study was aimed to assess the effect of proteolytic activity of Cur l 1 in augmenting allergic inflammation in mice. The induction of asthma like symptoms was confirmed by monitoring airway hyper-reactivity.

Materials and Methods

Fungal Antigen Preparation *C. lunata* was grown under stationary conditions in Sabouraud's broth for 13 days at 28°C. The spore mycelial extract was prepared in 50 mM NH₄HCO₃ (1/20; w/v) following the protocol described

earlier [21]. The extract was aliquoted, lyophilized, and stored at –80°C.

Enzyme Purification Cur l 1 was purified by a multi-step procedure, involving affinity chromatography on Concanavalin A Sepharose column followed by Fast protein liquid chromatography (FPLC) as per standardized protocol [21]. The purity of the protein was confirmed by SDS-PAGE analysis on 12% gel. Protein concentration was determined by Bradford assay (BioRad, CA). The isolated protein was used for further experiments.

Functional Activity of Cur l 1 and Enzyme Inactivation The enzymatic activity of isolated protein (Cur l 1) was checked using *N*- α benzoyl-L-arginine-ethyl ester hydrochloride (BAEE, Sigma, USA) assay as enzyme substrate. To obtain inactive protease, Cur l 1 was incubated with serine protease specific inhibitor (Phenyl methyl sulphonyl fluoride; 50 μ g PMSF/ μ g purified protein) for 30 min at 37°C. The inhibitor was removed by dialysis against saline for 24 h at 4°C. For determination of enzymatic activity, purified protein (3 μ g active or inactivated Cur l 1; 200 μ l) was incubated with 0.06 M BAEE (0.5 ml) in appropriate buffer (0.1 M Tris–HCl buffer, pH 7.0; 2.3 ml). The rise in absorbance at 253 nm (Δ OD₂₅₃)/minute/ μ g protein was recorded.

The enzymatic activity of Cur l 1 was confirmed by gelatin zymography [22]. For this active and inactive Cur l 1 were 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. The enzyme activity was visualized as a clear unstained region (band) in the gel against the blue background.

Animals and Groups Female Balb/c mice aged 4 to 6 weeks were obtained from Vallabhkhair Patel Chest Institute, Delhi. The mice were housed in the animal care facility of Institute of Genomics and Integrative Biology, Delhi under standard laboratory conditions. Mice were bled from the tail vein prior to start of the experiment to obtain pre-immune sera. The protocol for the study was approved by animal ethics committee of the institute.

Mice were randomly divided into five groups of six mice each. The control group (group 1) was sensitized and challenged with saline (0.9% NaCl). Ovalbumin (OVA) was administered to another group (group 2) as positive control. Groups 3, 4, and 5 mice were administered with Cur l 1 active protease, inactivated Cur l 1 protease and *Curvularia* extract, respectively.

Sensitization and challenge with antigens Immunization procedure described by Gough et al. was used for

sensitization and challenge with a slight modification [11]. The sensitization of mice was carried out intraperitoneally (i.p.) with 10 µg of OVA (Sigma, St. Louis, MO, USA), *Curvularia* extract or active Cur 11 in 100 µl saline adsorbed with 0.2 mg of aluminum hydroxide (Fig. 1a). Saline (100 µl) with alum was given to the negative control group. The day of sensitization was considered as day '0'. On day 14, mice were immunized i.p. and i.n. with 5 µg of OVA, *Curvularia* extract, active or inactive protease. Intranasal challenges were carried out with 2 µg of respective proteins on day 28, 29, and 30 in mild anaesthetized mice. The control group mice were challenged with 100 µl of saline alone. Airway hyper-responsiveness was recorded after 24 h of last challenge and mice were sacrificed using sodium pentobarbital (100 mg/kg i.p.). Blood, bronchoalveolar lavage fluid (BALF), spleen, and lung tissue were collected. Lung tissues from the right lobe were fixed for histological analysis and that from the left lobe was freeze dried to quantitate eosinophil peroxidase (EPO) activity.

Determination of Airway Hyper-responsiveness (AHR) Airway reactivity in response to methacholine challenge (MCh; Sigma, USA) was measured in conscious unrestrained mice in a preconditioned whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY, USA). Five mice from each group received nebulized PBS, followed by increasing concentrations of MCh (4, 8, 12, 16, 20, 24, and 50 mg/ml) to induce bronchoconstriction. Lung function was recorded and calculated as enhanced pause (penh). penh is a unitless parameter that correlates with pulmonary resistance [23]. Baseline penh value was recorded for 10 min.

Bronchoalveolar Lavage and Blood Collection Blood and bronchoalveolar lavage fluid (BALF) samples were collected as described by Ward et al. (1998). Mice were anaesthetized

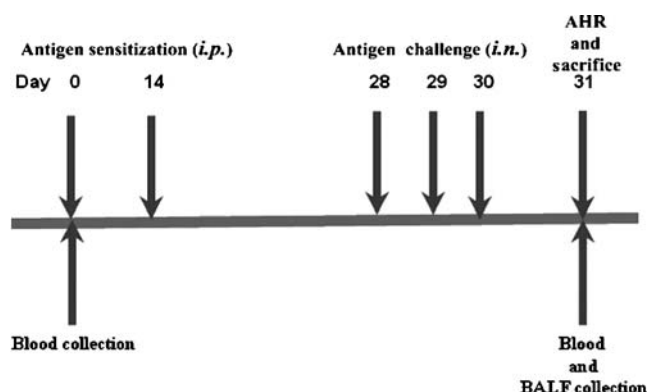


Fig. 1 Immunization protocol: Mice were immunized with antigens (Saline, Ovalbumin, *Curvularia* extract, active protease and inactive protease) on day 0 and 14 (i.p.). The mice were challenged intranasally on day 28, 29, and 30 with immunizing antigens

with sodium pentobarbital and blood samples were collected. The blood was allowed to clot for 2 h at room temperature prior to serum separation by centrifugation. The lungs were lavaged with two aliquots of 0.5 ml chilled 0.1 M PBS (pH—7.4) containing 5 mM ethylenediamine tetraacetic acid (EDTA). The BALF aliquots were pooled and stored on ice. BALF was centrifuged at 1,000×g for 15 min at 4°C, and the supernatant was separated and stored at –80°C. The cell pellet was resuspended in 1 ml of PBS and the total BALF cell counts were determined using the coulter counter (Coulter Corp., Miami, FL). Additionally, 100–150 µl of resuspended cells were spread onto glass slides. The BALF cells on glass slides were stained with Wright–Giemsa (Fisher Scientific) and eosinophils counted at 200 cells per slide (one slide per animal). The supernatant was used for determination of total protein content, cytokine levels and EPO activity.

Immunoglobulin Analysis The antigen specific IgE, IgG1, and IgG2a were measured in sera obtained from various groups of mice by indirect ELISA. Microtiter plates (Nunc, Denmark) were coated with 250 ng/100 µl/well of antigen in 0.1 M carbonate buffer (pH 9.6) for overnight at 4°C. The unbound antigens were washed with PBS and the unoccupied sites in the wells were blocked with 3% defatted milk in PBS, for 3 h at 37°C. Mice sera (IgE, 1:10; IgG1 and IgG2a, 1:50) were added (100 µl/well) and incubated overnight at 4°C. Pre-immune sera were also processed simultaneously. The plates were washed with PBST (PBS containing 0.05% Tween-20) and incubated for 3 h at 37°C with peroxidase conjugated anti-IgG1 and anti-IgG2a (1:1,000, BD Pharmingen, USA). For IgE detection, biotinylated anti-mouse IgE (BD Pharmingen, USA) was incubated at 25°C for 90 min. Following washing, the plates were incubated with streptavidin–peroxidase (1:1,000, BD Pharmingen, USA) for 30 min. The color was developed using *o*-phenylenediamine (OPD) and absorbance was read at 492 nm. ELISA for immunoglobulin levels was carried out on the same day.

Total Protein in BALF The protein content of BALF samples was estimated by Bradford assay following the manufacturer's instructions (BioRad, CA). The protein concentrations were extrapolated from a standard curve drawn using known concentrations of bovine serum albumin (BSA; Sigma, St. Louis, MO).

Lung Homogenate Preparation Lung homogenate was prepared following Schneider et al. for determination of eosinophil peroxidase activity (EPO) [24]. The lung tissue was homogenized (1:10 w/v) in HEPES buffer (50 mM, pH 8.0) using a pestle homogenizer (Werke, Germany). The suspension obtained was centrifuged and the pellet was

resuspended in same volume of 0.5% hexadecyl trimethyl ammonium bromide (HTAB) containing 5 mM EDTA. The process was repeated and the supernatant after centrifugation was collected and used for determination of EPO activity.

Eosinophil Peroxidase Activity EPO activity was determined following Cui et al. [25]. A volume of 50 µl of BALF supernatant or lung homogenate (1:10) was incubated with 100 µl substrate solution (3 mM *o*-phenylenediamine, 0.05 M Tris pH 8.0, 0.1% Triton X-100, and 8.8 mM H₂O₂) in the wells of microtiter plate (Tarsons, India). To differentiate the EPO activity from that of neutrophil and macrophage myeloperoxidases, activity was measured in absence or presence of EPO-specific inhibitor, resorcinol (1.5 mg/ml; Sigma, USA). The change in absorbance (492 nm) per minute was recorded using a spectramax ELISA reader (Molecular Devices, USA). The activity per microgram BALF protein or per gram dry weight of lung in each group was calculated as slope of linear rise in absorbance with time.

Splenocyte Culture Spleens from sacrificed mice were collected aseptically. Single-cell suspensions were prepared in complete Roswell Park Memorial Institute 1640 medium (RPMI, Invitrogen, UK) supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin. Spleen cells (1×10^6) from different groups of mice were added 100 µl per well of culture plate (Corning, US). The cells were challenged with 5 µg/ml of *Curvularia* extract, active or inactive protease (100 µl/well in RPMI). Splenocytes of OVA sensitized mice were stimulated with OVA. The cells were allowed to proliferate for 72 h at 37°C in a CO₂ incubator (Jouan, USA). The plates were centrifuged at 1,000 g, supernatant collected and used for cytokine analysis [12]. Cell pellet was washed with RPMI-1640 and incubated for 2 h with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 100 µg/ml). The cells were lysed in acidic isopropanol and absorbance was read at 570 nm. Optical density (OD) more than or equal to three times of the negative control was considered as positive stimulation index. Wells without antigen or with phytohemagglutinin (PHA, 5 µg/ml) were taken as negative and positive control, respectively.

Determination of Cytokine Levels The levels of IL-4, IL-5, and IFN-γ levels in BALF and splenocyte culture supernatants were estimated by sandwich ELISA following manufacturer's instructions (BD Pharmingen, USA). The detection range was 7.8–500 pg/ml, 15.6–1,000 pg/ml and 31.3–2,000 pg/ml for IL-4, IL-5, and IFN-γ, respectively.

Histopathology The lungs from sacrificed mice were fixed immediately in 10% neutral-buffered formalin (v/v) and

processed for histology. The lung tissue was embedded in paraffin, sliced to 4-µm sections and stained either with hematoxylin–eosin (HE) for analysis of lung inflammation or with periodic acid-Schiff (PAS) stain for the detection of mucus-producing cells. Sections were scanned under a light microscope and images were captured by a light microscope with an in-line camera (Nikon TE 2000-S, USA) and assembled into multipanel figures using Photoshop software (version 7.0, Adobe version). The degree of leukocyte infiltration around airways was graded as: 0—not present; 1—very few; 2—few; 3—moderate; 4—moderate to marked; and 5—marked. Goblet cell metaplasia in bronchial epithelium was scored based on number of PAS stained cells as grade 1: <20% cells stained; 2: 20–40% stained cells; 3: 40–60%; 4: 60–80%; and 5: >80% airways affected [26].

Statistical Analysis Student's *t* test was employed to compare the hydrolytic activity of Cur 1 1 active protease and PMSF treated Cur 1 1 (inactivated protease). One-way ANOVA with Bonferroni's multiple comparison test was performed using GraphPad Prism to examine differences in various parameters in groups of mice immunized with different antigens (GraphPad Prism software, San Diego California, USA, www.graphpad.com). *P*<0.01 was considered statistically significant.

Results

Inhibition of Proteolytic Activity In inhibition assay, active protease Cur 1 1 hydrolyzed BAEE and caused a change of 0.054 in absorbance units over 5 min. However, inactivated protease caused a change of 0.006 absorbance units in the same period showing >87% inhibition of proteolytic activity (Fig. 2a). The proteolytic activity of inactive Cur 1 1 was significantly lower than that of active Cur 1 1 (*p*<0.01). In qualitative assay (Zymogram), active Cur 1 1 showed a single band of proteolytic activity. In contrast, the lane containing inactive Cur 1 1 did not show any unstained region indicating lack of observable proteolytic activity (Fig. 2b).

Effect of Protease Activity on Methacholine-induced AHR AHR in antigen challenged mice was determined by measuring, penh value in response to incremental doses of methacholine (Mch). There was a dose dependent increase in airway reactivity to methacholine in different groups of mice (Fig. 3). Mice treated with *Curvularia* extract demonstrated maximum pause reflecting enhanced bronchoconstriction on challenge with methacholine, followed by active protease. In contrast mice challenged with inactive protease showed significantly lower AHR as

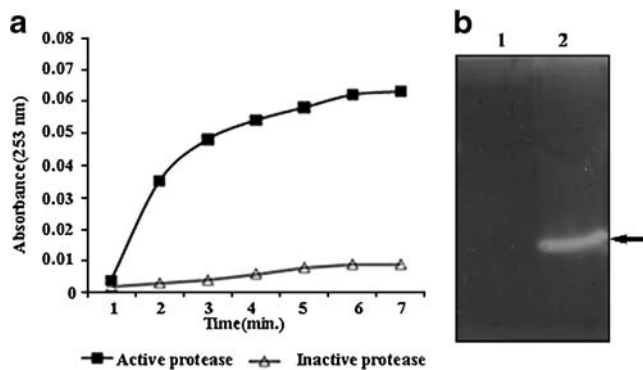


Fig. 2 **a** Quantitative estimation of hydrolytic activity of Cur 1 1: Active protease (Cur 1 1) and inactive protease (PMSF treated Cur 1 1) were assessed for their hydrolytic activity using BAEE as substrate. The proteolytic activity was presented as change in absorbance at 253 nm per unit time. Active and inactive protease showed significant difference in hydrolytic activity. **b** Qualitative assay of proteolytic activity: Active protease (Cur 1 1) and inactive protease (PMSF treated Cur 1 1) were assessed for their hydrolytic activity by gelatin zymography. The proteins were resolved on a 12% resolving gel copolymerized with 0.1% gelatin as substrate. The gel was stained with Coomassie brilliant blue and destained. Clear unstained region marked with arrow shows the proteolytic activity. Lane 1 Inactive protease, Lane 2 active protease

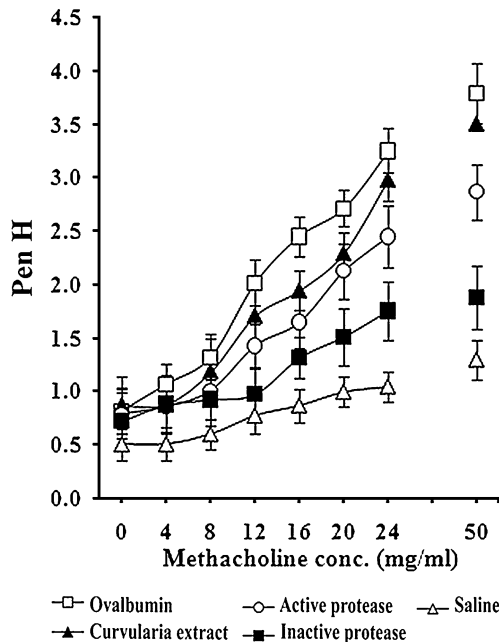


Fig. 3 Airway hyper-responsiveness (AHR) in response to active and inactive protease: The AHR was determined by whole-body plethysmography. Mice immunized with antigens (Saline, Ovalbumin, *Curvularia* extract, active protease and inactive protease) were exposed to increasing doses of methacholine (MCh). The airway resistance was recorded as penh value. Significantly higher AHR was recorded for active protease group than inactivated protease group

compared to OVA, active protease or *Curvularia* extract group mice ($p < 0.01$). The mice challenged with OVA showed significantly higher airway hyper-responsiveness in comparison to saline group.

Effect of Protease Activity on Induction of Serum Immunoglobulins Antigen specific IgE levels were highest in sera of *Curvularia*-immunized mice (0.518 ± 0.022) followed by active protease (0.510 ± 0.025) group mice. Specific IgE levels in active protease immunized mice were significantly higher as compared to inactivated protease group (0.250 ± 0.051). OVA group also showed high specific IgE levels (0.462 ± 0.042 , Fig. 4). Similar trend was observed for antigen specific IgG1 levels. *Curvularia*-immunized group showed maximum IgG1 level (0.468 ± 0.028) followed by active protease (0.405 ± 0.049). There was a significant difference in IgG1 levels ($p < 0.01$) between mice immunized with active protease and inactivated protease (0.190 ± 0.013). The IgG2a levels were similar for all the antigen-immunized mice. Immunoglobulin levels were lowest in saline control group mice.

Effect of Protease Activity on Cell Counts in Bronchoalveolar Lavage Fluid The cellular infiltration in BALF was highest in groups administered with OVA/*Curvularia* extract (Fig. 5). Active protease immunized and challenged mice showed maximum infiltration of cells $\{(13 \pm 1.323) \times 10^6\}$ followed by *Curvularia* extract $\{(11.87 \pm 0.264) \times 10^6\}$ and inactivated protease $\{(7.4 \pm 0.208) \times 10^6\}$ groups ($p < 0.01$).

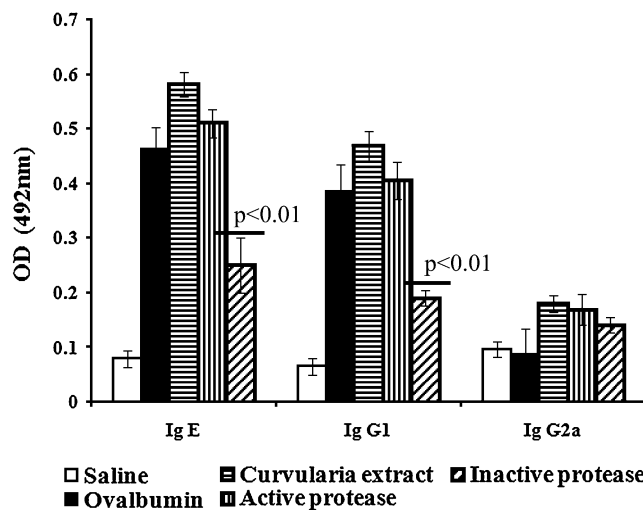


Fig. 4 Serum immunoglobulins: mice were immunized with various antigens (Saline, Ovalbumin, *Curvularia* extract, active protease and inactive protease) over a period of 30 days. Antigen specific IgE, IgG1, and IgG2a were measured in serum after last intranasal challenge by ELISA. Statistical difference in each parameter of active and inactive protease sensitized mice is mentioned as p value. $p < 0.01$ was considered statistically significant. Data are presented as mean $\pm$ SD. $n = 6$ mice per group

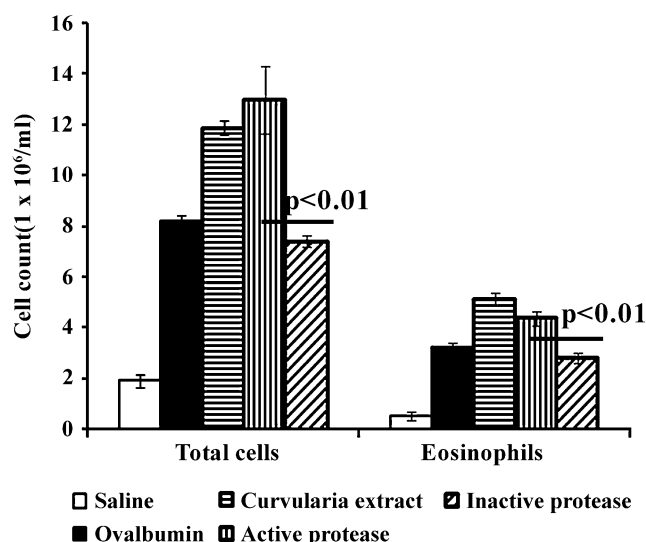


Fig. 5 Cellular infiltration in BALF: Mice were immunized with various antigens (Saline, Ovalbumin, *Curvularia* extract, active protease and inactive protease) over a period of 30 days. Total cell count was determined using hemocytometer. Eosinophils were identified based on the shape of nucleus in Wright–Giemsa stained slide. Statistical difference in each parameter of active and inactive protease sensitized mice is mentioned as *p* value. $p < 0.01$ was considered statistically significant. Data are presented as mean $\pm$ SD. $n = 6$ mice per group

There was a significant difference in cell counts between active protease and inactivated protease group mice ($p < 0.01$). The eosinophil count was maximum in *Curvularia* extract group $\{(5.12 \pm 0.234) \times 10^6\}$ followed by active protease group $\{(4.37 \pm 0.283) \times 10^6\}$ was significantly higher ($p < 0.01$) than inactivated protease mice $\{(2.8 \pm 0.193) \times 10^6\}$. OVA immunized mice showed high eosinophils count.

Occasional eosinophils were recorded in BALF obtained from saline control group mice.

Effect of Protease Activity on Protein Content of Bronchoalveolar Lavage Fluid The protein content was highest in BALF of mice immunized with *Curvularia* extract (0.383 ± 0.020 mg/ml). The protein content in BALF of active protease (0.36 ± 0.023 mg/ml) and inactivated protease challenged mice (0.232 ± 0.015 mg/ml) differed significantly ($p < 0.01$). The OVA immunized mice demonstrated high protein content (0.318 ± 0.020 mg/ml) whereas the protein content was lowest in PBS control mice (0.142 ± 0.008 mg/ml; Fig. 6).

Influence of Protease Activity on Activation of Eosinophils Eosinophil peroxidase activity (EPO) was measured as an indirect evidence for activation of eosinophils. EPO activity in BALF and in lung homogenate was higher in antigen-immunized groups in comparison to saline control mice (Fig. 6). Maximum EPO activity was recorded in BALF (227.33 ± 12.082) and lung homogenate (243 ± 20.602) of *Curvularia* extract group mice. Higher EPO activity was recorded in samples from active protease group (194 ± 20.148 in BALF and 204.33 ± 24.404 in lung homogenate) than those from inactivated protease group mice (97 ± 7.55 in BALF and 99.67 ± 12.89 in lung homogenate). EPO activity in both these groups differed significantly ($p < 0.01$). OVA immunization/challenge led to increase in EPO activity (123.67 ± 12.516 in BALF and 152.67 ± 33.501 in lung homogenate) in comparison to saline administration (49.66 ± 5.214 in BALF and 48.67 ± 7.082 in lung homogenate).

Splenocyte Proliferation on Stimulation with Antigens Spleen cells isolated from all antigen-immunized groups showed

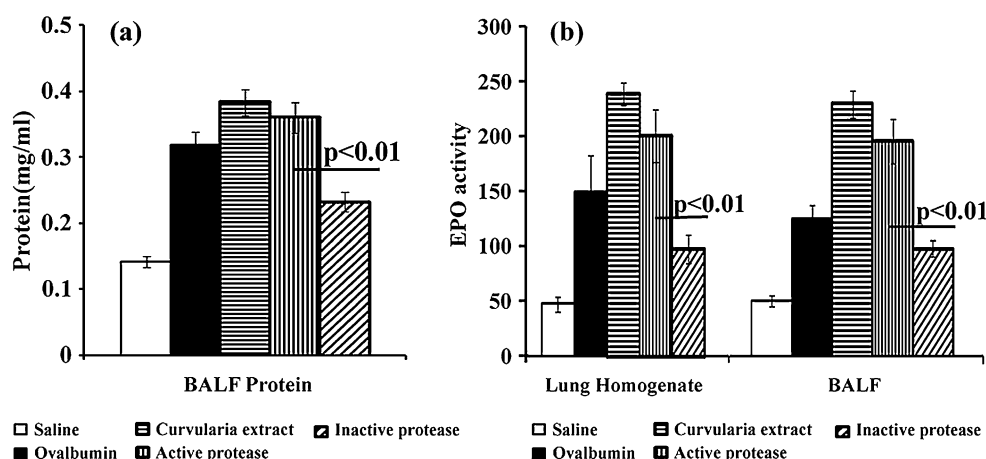


Fig. 6 **a** Total protein content in BALF: the protein content was estimated by Bradford assay following the manufacturer's instructions (BioRad, CA). **b** EPO activity in BALF and lung homogenate: The eosinophil peroxidase activity in BALF was measured per unit time per

milligram BALF protein. EPO activity in lung homogenate was measured per unit time per gram dry weight of lung tissue. Statistical difference in each parameter of active and inactive protease sensitized mice is mentioned as *p* value. $p < 0.01$ was considered statistically significant

positive stimulation indices in response to PHA. The proliferation in response to immunizing antigen was highest for *Curvularia* extract group followed by active protease group mice. Positive stimulation index was recorded for splenocytes from inactivated protease group mice on exposure with same antigen; but, there was a significant difference in proliferative response between active protease and inactivated protease group mice ($p<0.01$; Fig. 7). *Curvularia* extract, active protease and inactivated protease did not induce proliferation of splenocyte from OVA group and their stimulation index was similar to saline control.

Cytokine Analysis in BALF and Culture Supernatant (CS) Cytokine levels in both BALF (Fig. 8a) as well as splenocyte culture supernatant (Fig. 8b) showed variations in mice groups treated with different antigens. IL-4 levels were significantly elevated in *Curvularia* extract (78 ± 4 in BALF and 37.33 ± 2.082 in CS) than saline control mice (below detection limit). IL-4 level in active protease group (93.67 ± 4.163 in BALF and 53 ± 2.64 in CS) were significantly higher ($p<0.01$) in comparison to the levels in inactivated protease group (61.33 ± 8.676 in BALF and 35.67 ± 1.53 in CS). IL-5 levels were highest for active protease (105 ± 4 in BALF and 73.67 ± 1.845 in CS) followed by *Curvularia* extract (115.33 ± 4.163 in BALF and 68 ± 2.523 in CS) and inactivated protease group (82.67 ± 9.67 in BALF and 51.33 ± 1.527 in CS). The difference in IL-5

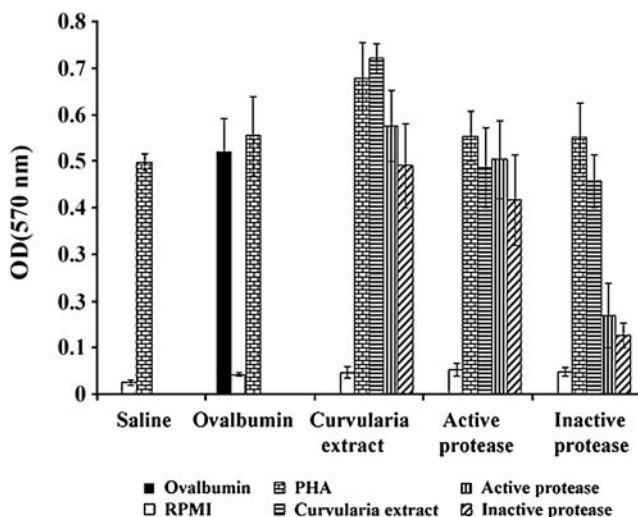


Fig. 7 Splenocyte proliferation by antigen stimulation: mice were immunized with various antigens (Saline, Ovalbumin, *Curvularia* extract, active protease and inactive protease) over a period of 30 days. Splenocytes of mice from different groups were stimulated with various antigens. RPMI and PHA were used as negative and positive control, respectively. The cells were incubated for 72 h at 37°C in a CO₂ incubator and the proliferation was measured by MTT assay. Statistical difference in stimulation index of active and inactive protease sensitized mice is mentioned as p value. $p<0.01$ was considered statistically significant

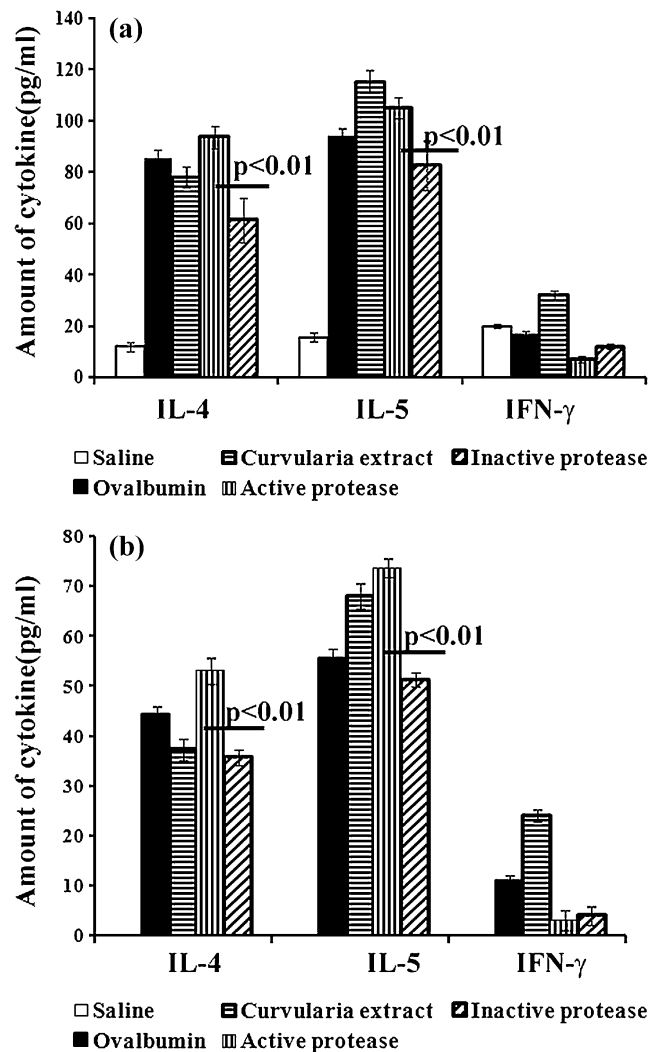


Fig. 8 Cytokine profile in (a) BALF and (b) splenocyte culture supernatant: IL-4, IL-5, and IFN-γ levels were determined by ELISA. Statistical difference in cytokine levels among active and inactive protease sensitized mice is mentioned as p value. $p<0.01$ was considered statistically significant. Data are presented as mean $\pm$ SD. $n=6$ mice per group

levels of active and inactivated protease mice was statistically significant ($p<0.01$). OVA immunized group also showed increased IL-5 levels (94.33 ± 2.855 in BALF and 55.67 ± 1.87 in CS) in comparison to saline group (12 ± 1.64 in BALF and below detection limit in CS). IFN-γ levels were low in BALF and CS and comparable for all the experimental groups including saline control.

Histological Analysis Histological analysis of lung sections was performed to assess the inflammatory effect of active or inactive protease. HE-stained lung tissue of *Curvularia* extract challenged mice showed numerous eosinophils into the lung interstitium around airways and blood vessels along with narrowing of airway lumen (Fig. 9a). Cellular infiltration and lumen narrowing were maximum in lung

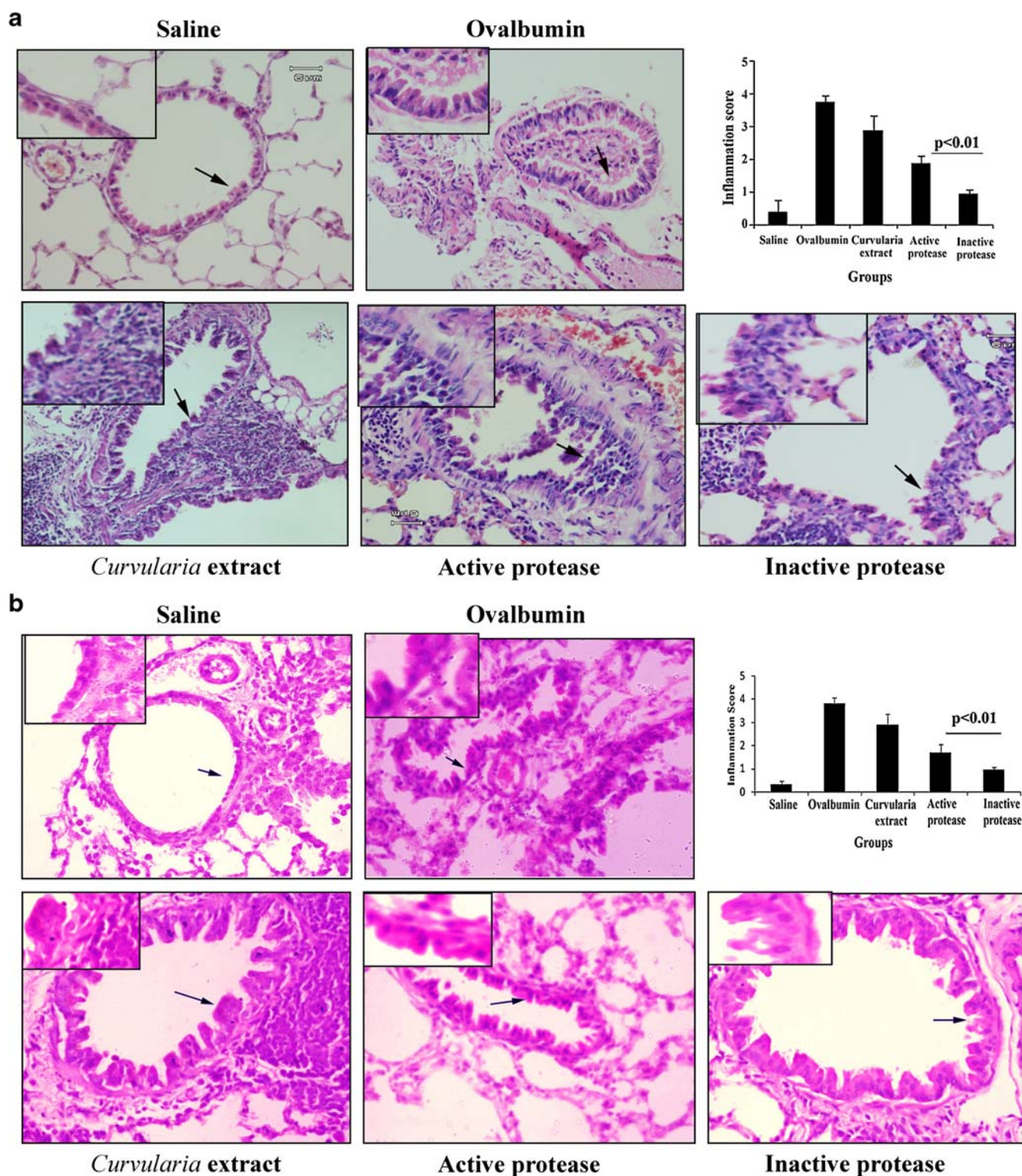


Fig. 9 a HE-stained sections of the lungs showing eosinophils infiltration (10×): Lungs were fixed with formalin, embedded in paraffin, sections cut and fixed on the slide for staining. Maximum cellular infiltration was observed in OVA group mice followed by *Curvularia* extract, active protease and inactive protease in order. The inflammation score was significantly higher in active protease than inactive protease group ($p < 0.01$). The inset on top shows the marked region at higher magnification (20×). Inflammatory cells are marked

by arrow. **b** PAS staining of lung tissues showing goblet cell hyperplasia and mucus secretion (10×): lungs were fixed with formalin, embedded in paraffin, sections cut and fixed on the slide for staining. OVA group mice had shown maximum mucus secretion followed by *Curvularia* extract, active protease which was significantly higher than inactive ($p < 0.01$). The inset on top shows the marked region at higher magnification (20×). Mucus layer marked by arrow

sections of active protease and OVA group mice. Lung sections from inactivated protease group mice showed significantly lower inflammatory score as compared to active protease group. The lung section from saline control group mice showed normal airway epithelia with occasional infiltrates (Fig. 9a).

Lung sections from OVA/*Curvularia* extract/active protease group mice showed narrowed airway lumens with enlarged epithelial cells and mucus plugs (Fig. 9B). The inactivated protease group mice showed lesser goblet cell hyperplasia in lungs. Only a few PAS stained epithelial cells were observed in lung tissue of saline control group mice.

Discussion

Hypersensitivity to inhalant allergens is associated with airway inflammation that contributes toward respiratory ailments like asthma and rhinitis. Despite extensive research on allergenic molecules; none of the structural attribute has been associated with allergies. However, few studies propose an alliance of functional features of certain enzymes with their allergenic activity. Concurrent with this, some studies show reduced inflammatory effect of allergens when their enzymatic activity is blocked [10, 11]. But none of these studies measured the actual effect of enzymatic activity on airway functioning. The present study was undertaken to evaluate the proteolytic effect of a major allergen (Cur l 1) on airway inflammation in mice.

Studies suggest that exposure to fungal allergens can lead to enhanced systemic immune response as well as response in the form of airway inflammation. The hyper-reactivity of airways is attributed to amplification of Th2 immune responses by allergen molecules. Whole-body plethysmography has been employed for assessment of airway functioning in various studies [27]. In the present study, plethysmography was used to compare the Mch-induced AHR in Cur l 1 active protease and Cur l 1 inactivated protease group mice. There was a significant difference in penh values among active protease and inactivated protease group mice ($p < 0.01$). The enhanced airway reactivity in active protease group mice indicates an influence of functional activity of this enzyme. Since Th2 milieu is a prime contributor to airway hyper-reactivity, the parameters like serum immunoglobulins and Th2 cytokines were assessed among different antigen groups.

IgE plays an instrumental role in development of allergic hypersensitivity. Literature suggests that cysteine protease activity of Der p 1 and Der f 1 biases the immune response in favor of IgE by modulating the Th2 and IgE regulation [28]. Der p 1 cleaves surface molecule CD23 from B cells and enhances IgE production by ablating an inhibitory feedback mechanism that normally limits IgE synthesis

[29]. Cleaved CD23 results in increased soluble CD23 that increases interleukin IL-4-mediated IgE production [29]. In the present study, proteolytically active protein augmented the IgE synthesis significantly higher as compared to inactivated protease. This augmentation may be attributed to the difference in protease activity of the protein.

Proteases from house dust mite are capable of cleaving the IL-2 receptor (CD25) from T cells, blocking a major Th1 cytokine that results in increased IL-4 and less IFN- γ from T lymphocytes [30]. Dust mite protease allergens are also reported to stimulate keratinocytes and airway epithelial cells to release cytokines and chemokines that may be involved in the development of allergy. In the present study, active Cur l 1 immunization/challenge increased the IL-4 and IL-5 levels significantly in BALF and splenocyte culture supernatant compared to inactivated protease. This suggests important contribution of proteolytic activity of Cur l 1 in enhancing the Th2 response. The enhancement of IL-4 and IL-5 cytokines in *Curvularia* extract immunized group may be attributed to the presence of proteases and other allergens. The IFN- γ , a Th1 cytokine representative remained unchanged in all the antigen groups.

Kauffman has reviewed the effect of proteases from airborne allergens on the respiratory mucosa [31]. It was proposed that pro-allergic environment induced by proteases may be due to activation of protease activated receptors (PARs) which are a family of G-protein coupled receptors. In a study, serine proteases of house dust mite (Der 3 and Der p 9) were shown to activate one such receptor, PAR-2. The induction of inflammatory cytokines (IL-6 and IL-8) and chemokines in animals exposed to these proteases was attributed to activation of PAR-2 [14]. PAR-2 dependent pathways have been proposed for chemokines production in protease laden fungal extract induced epithelial cells [31]. Expression of PARs has also been reported to influence airway hyper-reactivity [32]. In the present study, Cur l 1 sensitized mice showed enhanced allergic response and airway hyper-reactivity. Though the exact mechanism by which Cur l 1 augments allergic response is not clear, activation of PAR is one of the probable mechanisms.

Der p 1 has been characterized as a cysteine protease, and this activity has been confirmed in Group 1 allergens from the other species of mites that cause allergy. Group 3 mite allergens are serine proteases. Additionally, mite extracts contain elastase, chymotrypsin-like protease, and other proteases. Mite proteases increase vascular permeability and detach epithelial cells [33]. Proteases from bacterial and dust mite sources can cause epithelial disruption and proteolysis. This proteolysis leads to plasma exudation and cellular infiltration. The results of the present study showed significantly higher cellular infiltration including eosinophils in active protease group than inactivated protease

mice. The raised protein content, EPO activity and mucus deposition in active protease challenged mice suggest the important role of proteolytic activity in mediator release and subsequent airway inflammation. Enzymatically inactivated Cur l 1 showed lowest cellular infiltration, protein content and EPO activity among the antigens tested.

Crude extracts from several fungi, namely *Aspergillus fumigatus*, *Epicoccum purpurascens*, *Penicillium* spp. etc. have been shown to harbor proteases [13, 15–17]. The proteases of fungal extract are proposed to influence inflammatory response. Further, such extracts contain multiple allergenic components. In the present study, administration of crude *Curvularia* extract to mice led to induction of strong pro-allergic response. This may be attributed to presence of multiple allergens (including several proteases) in *Curvularia* extract.

Proteases have been demonstrated to influence production of immunoglobulins, activate eosinophils and neutrophils, facilitate their penetration into the bronchoalveolar space and enhance permeation of inflammatory cells through respiratory epithelia [9]. Under physiological conditions, a homeostatic level of internal proteases is achieved. Exposure to extrinsic proteases can rupture the epithelial barriers. In the present study, we have evaluated the importance of proteolytic activity of Cur l 1 in exacerbation of airway inflammation using murine model of asthma. In conclusion, the biological activity, i.e., proteolytic nature of Cur l 1 plays an important role in exacerbation of airway inflammation. The inactivated counterpart may be explored for immunotherapy of allergic diseases.

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