Chapter 4 Proinflammatory effects of alkaline serine protease

INTRODUCTION

Hypersensitivity to inhaled bioparticles is often the cause of airway inflammation in predisposed individual. Studies suggest that sustained inflammation of airways is followed by structural changes that cause deterioration in respiratory function (Lloyd and Robinson, 2007). The pathogenesis and persistence of allergic diseases including asthma is influenced by exposure to airborne allergens. Allergen(s) interaction with specific IgE triggers mast cell degranulation, lymphocyte activation and release of proinflammatory cytokine. This advances to eosinophilic and other cell infiltration, and usually culminates in allergic airway disease. Thus it is important to evaluate the effects of potential components/allergens in airway inflammation and their impact on pathology of bronchial hyperresponsiveness. Previously allergenic extracts of fungi were shown to induce morphological changes, cell desquamation and production of proinflammatory cytokines in alveolar epithelium using cell line (Kauffman et al., 2000). The changes induced were attributed to proteases present in the extracts since the effects were abolished upon inhibition of proteases. This approach allowed assessment of changes induced but it did not reveal the exact mechanism underlying sensitization and pathogenesis of disease.

The in vivo approach involves experimental introduction of protein into animal system. Animal models that simulate human asthma, allow thorough functional assessment of the molecule and the underlying processes that contribute to asthma (Szelenyi, 2000). Most in vivo models of allergic airway diseases have employed murine system due to availability of strains with well-characterized immune system and detailed knowledge of the murine biology and reagents. The models of allergic airway disease show effect in terms of cellular infiltration of airways, upregulated IgE synthesis and cytokine release, increased mucus production, collagen deposition/airway remodeling and bronchial hyperresponsiveness (Tanaka et al., 2001).

Murine models developed with fungal allergens or spores show characteristic feature of asthma such as airway narrowing, goblet cell hyperplasia, eosinophil recruitment into the airways and Th2 type milieu Havaux et al. (2005)
demonstrated that Alternaria alternata and Cladosporium herbarum spores have the ability to induce a Th2 antibody response characterized by a strong production of polyclonal IgE antibodies and specific IgG (Havaux et al., 2005). Furthermore, pulmonary exposure to Aspergillus fumigatus results in severe allergic response characterized by enhanced total serum IgE, Th2 cytokine levels, bronchial hyperresponsiveness and airway remodeling (Schuh et al., 2002). Fungi, owing to the presence of proteases, are well equipped for inducing airway inflammation and remodeling process. A recent study reported strong allergic inflammatory response was recorded in a murine model upon sensitization to protease allergens released by viable P. chrysogenum conidia (Schwab et al., 2004). However, the protease rich fungal spore extract was employed in this study and the actual effect of protease alone could not be evaluated. Kheradmand et al. employed specific inhibitors of proteases (in case of allergen extract) as well as purified fungal protease for induction of allergic lung inflammation in murine model and demonstrated that protease content influences the disease induction and severity (Kheradmand et al., 2002). Furthermore, study showed that proteases, due to their functional activity, can overcome airway tolerance and induce pulmonary disease. They categorized fungal proteases as type II allergens i.e. which have intrinsic ability to bypass induction of airway tolerance and thus can induce inflammation without an additional adjuvant. In contrast, allergens like ovalbumin were categorized as type I i.e. which do not have such intrinsic capability. However, the study used protease in combination with a non-enzymatic allergen and focused on evaluation of the adjuvant properties of the protease (Kheradmand et al., 2002). Studies have established the role of proteases in fungal extracts in mediating allergic inflammation (Schwab et al., 2004; Shin et al., 2006; Tai et al., 2006). However, the role of biochemical activity of these proteases in the pathology of allergic inflammation remains to be elucidated.

Various parameters such as route, time of exposure and dose of protease allergen(s) were considered for sensitization of mice to develop airway disease model. Most protocols involve giving an initial sensitization with allergen followed by a time lag for adaptive response to develop. Both systemic and local exposures have been employed to initiate sensitization. A dual route allergen administration (i.p.
and *i.n.*) was more effective in inducing airway hyperresponsiveness and airspace eosinophilia in Balb/c mice than immunization by intraperitoneal route alone (Zhang et al., 1997). Gough et al. employed a combination of intraperitoneal and subcutaneous routes to induce the allergic response in mice (Gough et al., 2003). The combination of these two routes allowed both, rapid absorption (*i.p.*) and slow absorption (*s.c.*). One or more challenges by the respiratory route (inhalation, intratracheal exposure, intranasal treatment or aspiration) are usually employed to achieve optimum inflammation.

Besides route and timing, allergen dose also influences the allergic response. The critical effect of different allergen dose has been emphasized in recent studies. The high-dose exposure to cat allergen resulted in reduced IgE response to Fel d 1, while low-dose exposure to Fel d 1 posed the strongest risk for sensitization (Woodfolk, 2005). Thus, an optimal dose and appropriate protocol/schedule were selected for the present study to develop an *in vivo* model of airway inflammatory disease. A dose of 10 µg protein (protease) given by two different routes was tested for effective route leading to allergic inflammation in mice (Scwab et al., 2004).

*C. lunata* is among the predominant airborne molds sensitizing 10-16% of atopic individuals worldover and inducing allergic diseases like allergic rhinitis and asthma (Travis et al., 1991; Horner et al., 1995; Gupta et al., 1999). Further, *C. lunata* shares six IgE reactive proteins of 26, 31, 38, 45, 50 and 78 kDa with other *Curvularia* species (Gupta et al., 2002). A protease allergen of 31 kDa from *C. lunata* spore-mycelial extract was isolated by Con-A Sepharose and gel filtration chromatography (Gupta et al., 2004). The N terminus sequence of the protein showed 92% identity to *A. oryzae*, *A. fumigatus* and *A. flavus* alkaline serine protease precursor. The 31 kDa allergen showed protease activity on different substrates and was designated as Cur l 1 (Gupta et al., 2004). The present study investigated the effect of proteolytic activity of Cur l 1 in inducing allergic inflammation in mice. The induction of asthma like symptoms in mice model was demonstrated by airway hyperreactivity.
MATERIALS AND METHODS

**Antigen preparation:** C. lunata was grown under stationary conditions in Sabouraud’s broth for 13 days at 28°C. The antigens were extracted from spore mycelial powder in 1/20 w/v 50 mM NH₄HCO₃ (Gupta et al., 2004). The extract was lyophilized in small aliquots and stored at -80°C.

**Enzyme purification:** Spore-mycelial extract (protein) of C. lunata was fractionated for purification of Cur l 1 affinity chromatography on Concavalin A Sepharose column followed by Fast protein liquid chromatography (FPLC) as described earlier (Gupta et al., 2004). The purity of the protein was confirmed by SDS-PAGE analysis on 12 % gel. Protein concentration was determined by bicinchoninic acid assay (BCA, Sigma, USA). The protein isolated was used for immunochemical experiments.

**Functional activity of Cur l 1 and enzyme inactivation:** The enzymatic activity of protein (Cur l 1) was checked using Nα-benzoyl-L-arginine-ethyl ester hydrochloride (BAEE, Sigma, USA) as enzyme substrate. To obtain inactive protease (CIAP), Cur l 1 was incubated with serine protease specific inhibitor (Phenyl methyl sulphonyl fluoride; 50 μg PMSF / μg purified protein) for 30 minutes at 37°C. The inhibitor was removed by dialysis against saline for 24 hours at 4°C. For determination of enzymatic activity, purified protein (3 μg active or inactive Cur l 1; 200 μl) was incubated with 0.06 M BAEE (0.5 ml) in Tris-HCl buffer (0.1 M Tris-HCl buffer, pH 7.0; 2.3 ml). Rise in absorbance at 253 nm (ΔOD₂₅₃)/ minute/μg protein was recorded.

The enzymatic activity of Cur l 1 was confirmed by gelatin zymography (Heussen and Dowdle, 1980). 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 Coomassie brilliant blue (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 Vallabhbhai 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 Ethical Committee of the institute. Mice were randomly divided into five groups with 6 mice each. The control group (group 1) was sensitized and challenged with saline (0.9% NaCl). Ovalbumin (OVA, Sigma, St. Louis, MO, USA) was administered to another group (group 2) as a positive control. Group 3, 4 and 5 were administered with Cur l 1 active protease (CAP), inactive Cur l 1 protease (CIAP) and *Curvularia* extract (CLE), respectively (Figure 4.1).

**Sensitization and challenge with antigens:** Immunization procedure as described by Gough et al. was used for the present study with a slight modification (Figure 4.1). The sensitization of mice was carried out intraperitonely with 10 μg of OVA, *Curvularia* extract or active Cur l 1 in 100 μl saline adsorbed with 0.2 mg of aluminum hydroxide. Saline (100 μl) with alum was given to the negative control group. The day of initial sensitization (*i.p.*) 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 protein. Intranasal challenges were carried out with 2 μg of respective protein(s) on day 28, 29 and 30 in mild anaesthetized mice. The control group was challenged with 100 μl of saline. Airway hyperresponsiveness was recorded after 24h of last challenge and mice were sacrificed using sodium pentobarbital (100 mg/kg *i.p.*). On day 31 blood, bronchoalveolar lavage fluid (BALF), spleen and lung tissue were collected. Lung tissue from the right lobe was fixed for histological analysis and that from the left lobe was freeze dried to quantitate eosinophil peroxidase (EPO) activity.
Figure 4.1: 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.

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Determination of airway hyperresponsiveness (AHR): Airway reactivity in response to methacholine (MCh; Sigma, USA) challenge was measured in conscious unrestrained mice in a preconditioned whole-body plethysmograph (Buxco Electronics, Inc., Troy, NY, USA). Five mice from each group received nebulised 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 (Schneider et al., 1997). Baseline Pen H value was recorded for 10 minutes.

Bronchoalveolar lavage and blood collection: Blood and bronchoalveolar lavage fluid (BALF) samples were collected as described in chapter 2. Blood samples were collected, serum separated by centrifugation, and stored at −70°C. The lungs were lavaged with 1 ml aliquots of PBS twice. The BALF aliquots were pooled and centrifuged at 100×g for 15 min at 4°C, the supernatant was separated and the cell pellet was resuspended in 1ml PBS. Total cell counts in BALF were made using coulter counter (Coulter Corp., Miami, FL). Additionally, 100 µl of cells were spread onto glass slides. The BALF cells on glass slides were stained with Wright-Giemsa (Fisher Scientific) stain and eosinophils counted approximately 200 cells per slide (one slide per animal). The BALF supernatant was used for determination of total protein content, cytokine levels and eosinophil peroxidase (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) by overnight incubation at 4°C. The unbound antigen was 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 was also added in separate wells and 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
(1:1000, BD Pharmingen, USA). For IgE detection, biotinylated anti-mouse IgE (1:1000, BD Pharmingen, USA) was incubated at 25°C for 90 min. Following washing, the plates were incubated with streptavidin-peroxidase (1:1000, BD Pharmingen, USA) for 30 minutes. The colour was developed using o-phenylenediamine 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 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) (Schneider et al., 1997). The lung tissue was homogenized (1:10 w/v) in N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (50 mM, pH 8.0) using a pestle homogenizer (Werke, Germany). The suspension obtained was centrifuged and the pellet was re-suspended in same volume of 0.5% hexadecyl trimethyl ammonium bromide (HTAB) containing 5 mM ethylenediamine tetraacetic acid (EDTA). The process was repeated, supernatant was collected after centrifugation and used for determination of EPO activity.

**Eosinophil peroxidase activity:** EPO activity was determined following Cui et al. (Cui et al., 2003). 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 microtitre 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 x 10^6) from different group mice were added 100 μl per well of culture plate (Corning, USA). The cells were challenged with 5 μg/ml of *Curvularia* extract, enzymatically active or inactive protein (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 CO_2_ incubator (Jouan, USA). The plates were centrifuged at 1000 g, supernatant collected and used for cytokine analysis (Tai et al., 2006). 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) ≥ 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 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) and explained in chapter 2. The detection range was 7.8-500 pg/ml, 15.6-1000 pg/ml and 31.3-2000 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 with an in-line camera (Nikon TE 2000-S, USA) and assembled into multipanel figures using Photoshop software (version 7.0, Adobe version). The 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: 0-20 % cells stained; 2: 20-40 % stained cells; 3: 40-60 %; 4: 60-80 % and 5: >80% airways affected (Inoue et al., 2007).

**Statistical analysis:** Student’s t-test was employed to compare the hydrolytic activity of Cur l 1 active protease and PMSF treated Cur l 1 (inactive protease). One-way ANOVA with Bonferroni’s multiple comparison test was performed using GraphPad Prism to examine differences in various parameters in 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 hydrolysed BAEE and caused a change of 0.054 in absorbance units over 5 minutes. However, inactive protease caused a change of 0.006 absorbance units in the same period showing >87 % inhibition of proteolytic activity (Figure 4.2a). The proteolytic activity of inactive Cur l 1 was significantly lower than that of active Cur l 1 (\( p<0.01 \)).

In qualitative assay (Zymogram), active Cur l 1 showed a single band of proteolytic activity. In contrast, the lane containing inactive Cur l 1 did not show any unstained region indicating lack of proteolytic activity (Figure 4.2b).

**Effect of protease allergen on methacholine induced AHR:** After antigen challenge AHR was determined by measuring PenH value in response to incremental doses of methacholine (MCh). There was a dose dependent increase in AHR to methacholine challenge in all groups of mice (Figure 4.3). Mice treated with Curvularia extract demonstrated maximum pause reflecting enhanced sensitivity to challenge with methacholine, followed by active protease. In contrast mice challenged with inactive Cur l 1 showed lower AHR as compared to OVA, active protease or Curvularia extract.
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Figure 4.2a: Quantitative estimation of hydrolytic activity of Cur l 1: Active protease (Cur l 1) and inactive protease (PMSF treated Cur l 1) were assessed for their hydrolytic activity using BAEE as substrate. The proteolytic activity was assessed as change in absorbance at 253nm per unit time. Active and inactive protease showed significant difference in hydrolytic activity.

Figure 4.2b: Qualitative assay of proteolytic activity: Active protease (Cur l 1) and inactive protease (PMSF treated Cur l 1) were assessed for their hydrolytic activity by gelatin zymography. The proteins were resolved on a 12% resolving gel co-polymerized 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.
Figure 4.3: Airway hyperresponsiveness (AHR) in response to active and inactive protease: The AHR was determined by whole body plethysmography. Mice challenged 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 Pen H value. Significantly higher AHR was recorded for active protease group than inactive protease group.
(CLE) group mice (p<0.01). The mice challenged with OVA showed significantly higher (p<0.01) airway hyperresponsiveness in comparison to saline group.

**Effect of protease allergen on induction of serum immunoglobulins:** Highest antigen specific IgE levels were recorded in sera of *Curvularia* extract immunized mice (0.581±0.022) followed by active protease (0.51±0.025) group mice. Specific IgE levels in active protease group mice were significantly higher as compared to inactive protease group (0.250±0.051). OVA group mice also showed high specific IgE levels (0.462±0.042) compared to saline group (Fig. 4.4). Similar trend was observed for antigen specific IgG1 levels. *Curvularia* extract 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 between groups immunized with active protease and inactive protease (0.190±0.013, p<0.01). The IgG2a levels were however, similar for all the groups of mice. Low immunoglobulin levels were recorded in saline control group.

**Effect of protease allergen on cell counts in bronchoalveolar lavage fluid:** The cellular infiltration in BALF was highest in groups administered with OVA/*Curvularia* extract (Fig. 4.5). active protease immunized and challenged mice showed maximum infiltration of cells {(13±1.323)×10^6} followed by *Curvularia* extract {(11.87±0.264)×10^6} and inactive protease {(7.4±0.208)×10^6} groups. There was a significant difference in total cell counts between active protease and inactive protease mice (active protease>inactive protease, p<0.01). The eosinophil count was maximum in *Curvularia* extract group {(5.12±0.234)×10^6} followed by active protease group mice. The eosinophil count in active protease group {(4.37±0.283)×10^6} was significantly higher than that in inactive protease group mice {(2.8±0.193)×10^6}(p<0.01). OVA immunized mice showed high eosinophils counts whereas, occasional eosinophil counts were recorded in BALF obtained from saline control group.
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**Figure 4.4: Serum immunoglobulins:** Mice were challenged 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 by ELISA after last intranasal challenge. 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 is presented as mean ± SD. n=6 mice per group.

**Figure 4.5: Cellular infiltration in BALF:** Mice were challenged with various antigens (Saline, Ovalbumin, *Curvularia* extract, active protease and inactive protease) over a period of 30 days. Total cell count was determined using haemacytometer. Eosinophils were identified based on the shape of nucleus in Wright-Giems 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 ± SD. n=6 mice per group.
**Chapter 4**

**Proinflammatory effects of alkaline serine protease**

**Effect of protease allergen on bronchoalveolar fluid protein content:** 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 immunized mice (0.36±0.023 mg/ml) and inactive protease immunized mice (0.232±0.015 mg/ml) differed significantly (p <0.01). The OVA immunized mice demonstrated high protein content in BALF (0.318±0.020 mg/ml). The protein content was lowest in PBS control mice (0.142±0.008 mg/ml) (Figure 4.6a).

**Influence of protease allergen on activation of eosinophils:** The activity of enzyme eosinophil peroxidase was measured as indirect evidence for activation of eosinophils. The EPO activity in BALF and in lung homogenate was higher in antigen immunized groups in comparison to saline control mice (Figure 4.6b). Maximum EPO activity was recorded in in BALF (227.33±12.082) and lung homogenate (243±20.602) of *Curvularia* extract immunized mice. Higher EPO activity was also recorded in BALF (194±20.148) and lung homogenate (204.33±24.404) samples obtained from active protease group mice. The EPO activity was lower in BALF (97±7.55) and lung homogenate (99.67±12.89) of inactive protease group mice. The EPO activity in active protease and inactive protease group differed significantly (p<0.01). OVA immunization led to significant increase in EPO activity in BALF (123.67±12.516) and lung homogenate (152.67±33.501) compared to saline group mice (49.66±5.214 in BALF and 48.67±7.082 in lung homogenate).

**Splenocyte proliferation on stimulation with protease allergen:** Spleen cells isolated from antigen immunized groups showed positive stimulation indices in response to PHA. The proliferation in response to immunizing antigen was highest for *Curvularia* extract group mice followed by active protease group mice. Positive stimulation index was recorded for splenocytes from inactive protease in response to inactive protease. However, there was a significant difference in proliferative response to immunizing antigen in active protease and inactive protease group (p<0.01) (Figure 4.7). *Curvularia*
Figure 4.6: (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.
Figure 4.7: Splenocyte proliferation by antigen stimulation: Mice were challenged 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 72h 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 exposed mice is mentioned as p-value. p < 0.01 was considered statistically significant.
extract, active protease and inactive protease did not induce proliferation of splenocyte from OVA group.

**Cytokine release on stimulation with protease allergen:** Cytokine levels in both BALF (figure 4.8a) as well as splenocyte culture supernatant (Figure 4.8b) showed variations in mice groups immunized and challenged with different antigens. IL-4 levels in BALF (78.00±4.00) and CS (37.33±2.08) were enhanced in Curvularia extract immunized mice than other groups. IL-4 level in active protease group mice (93.67±4.163 in BALF and 53±2.64 in CS) were significantly higher in comparison to the levels in inactive protease group (61.33±8.676 in BALF and 35.67±1.53 in CS) (p<0.01). The level of IL-4 in CS was below detection limit in saline group mice. IL-5 levels were highest in active protease group (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 inactive protease group mice (82.67±9.67 in BALF and 51.33±1.527 in CS). The difference in IL-5 levels of active protease and inactive protease group mice was statistically significant (p<0.01). OVA immunized group also showed increased IL-5 levels in BALF (94.33±2.855) and CS (55.67±1.87) compared to saline group mice (12±1.64 in BALF and below detection limit in CS). The IFN-γ levels were low in BALF and CS of all the experimental group mice including saline control.

**Histological analysis of lung tissue:** 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 (Figure 4.9a). High degree of cellular infiltration and narrowing of lumen were observed in lung sections of active protease and OVA immunized mice than saline control. Lung sections of inactive protease mice showed significantly (p<0.01) lower inflammatory score as compared to lung sections of active protease group mice. The lung section of saline group mice showed normal airway epithelia with occasional cellular infiltrates (Figure 4.9a).
Lung sections from OVA/\textit{Curvularia} extract/active protease immunized mice showed narrowed airway lumen with enlarged epithelial cells and mucus plugs (Figure 4.9b). The lung sections of inactive protease group mice showed lesser degree of goblet cell hyperplasia than active protease. In saline control group, a few PAS stained epithelial cells were observed.
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Figure 4.8: 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 is presented as mean ± SD. n=6 mice per group.
Figure 4.9a: HE stained sections of the lungs showing eosinophils infiltration (10x): 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. The inflammation score was significantly higher in active protease than inactive protease group mice (p<0.01). The inset on top shows the marked region at higher magnification (20x). Inflammatory cells are marked by arrow.
Lungs were fixed with formalin, embedded in paraffin, sections cut and fixed on the slide for staining. OVA group mice showed maximum mucus secretion followed by *Curvularia* extract and active protease which was significantly higher than inactive protease group (p<0.01). The inset on top shows the marked region at higher magnification (20x). Mucus layer is marked by arrow.
Discussion

Allergens with enzymatic activity have been isolated and identified from a variety of sources. Hydrolytic enzymes such as trypsin, chymotrypsin, lipases, pepsin, bromelain, papain and cellulase are implicated in occupational asthma (Thompson, 1998). Apart from these, cysteine and serine proteases from house dust mite (Der p 1, Der p 3, and Der p 9), serine proteases of *Aspergillus* and *Penicillium* species and phospholipase A 2 of bee venom are clinically significant domestic allergens. Most of these allergens have high potency, hence immunological response to these allergens is armed by their intrinsic biological activity (Pomes et al., 2002). Owing to the high allergenic potency, the response to such allergens is more pronounced towards immune inflammation and can be studied using appropriate animal model. In the present study, effects of active and inactive serine protease (Cur l 1) of *C. lunata* were evaluated, using mouse model. The proinflammatory effects of Cur l 1 were also compared with *Curvularia* allergen extract. Here, ovalbumin and saline immunized mice served as positive and negative control, respectively.

Studies show that protease allergens can disrupt the homeostatic control of IgE levels by cleaving regulatory molecules such as CD23 on B-cells (Hewitt et al., 1995). Hence enhanced production of IgE occurs in mice exposed to external proteases. *In-vitro* studies with Der p 1 have shown that active proteases condition dendritic cells to express more CD86 and less CD40, thereby enabling them to stimulate the differentiation of naive T cells towards the Th2 phenotype. Thus, the microenvironment conducive for IgE production is created (Ghaemmaghami and shakib, 2002). In the present study, Cur l 1, a 31 kDa protease of *C. lunata* induced high specific IgE and IgG1 levels in mice. The enzymatically inactive Cur l 1 however, showed lower IgE and IgG1 response compared to active protease. This may be due to Th2 environment created by proteolytic activity of allergen. However, the levels of both IgE and IgG1 in inactive protease challenged group were comparatively higher than the control. This shows that inactive protease retains allergenicity probably due to its structural epitopes.
The allergenic potential of antigens is assessed based on their ability to direct secretion of inflammatory cytokines like IL-4, IL-5 and IL-13. *In vitro* studies with Der p 1 allergen show that it can cleave CD-40 from dendritic cell surface causing reduced IL-12 production. This signals increase in IL-4 (Th2) and reduction in IFN-γ (Th1) production. In the present study, active protease and *Curvularia* extract immunized mice showed a bias towards Th2 milieu. IL-4 levels were elevated in BALF and splenocytes culture supernatant of mice challenged with active protease. Mice immunized with inactive protease also showed increased IL-4 levels in comparison to saline group mice. It shows that inactive Cur 1 retains allergenicity but the potency of inactive protease is attenuated. The elevated IL-4 levels may also account for increased IgE in active protease group mice since IL-4 directs the recombination to constant ‘’ chain of immunoglobulin (Correy and Kherandmand, 1999). The levels of IFN-γ, representative of Th1 response were however, low in all the antigen treated mice.

*In vitro* studies have shown that protease molecules can cause epithelial cell disruption and proteolysis (Wan et al., 2000; 2001). Further, such molecules may proteolytically cleave tight junction protein ‘occludin’ causing increased epithelial permeability (Tai et al., 2006). Exposure to such proteases by inhalation may thus induce plasma exudation and cellular infiltration. In the present study, protein content in BALF was measured as a non specific marker of oedema. Increased protein levels in active protease immunized group mice may be due to increased vasculature and bronchoalveolar permeability caused by the active protease allergen.

Chronic asthma is associated with an airway wall remodelling including epithelial cell hyperplasia (increased number), goblet cell metaplasia, thickening of basement membrane, sub-epithelial fibrosis and increased airway smooth muscle mass (Havaux et al., 2005; Tai et al., 2006). Histological examination of lungs of active protease and *Curvularia* extract immunized mice showed increased peribronchial and perivascular infiltration. The inflammatory response in *Curvularia* extract and active protease immunized mice was comparable to that induced by ovalbumin. In comparison, the levels of cellular infiltration were moderate in inactive protease challenged mice. The
total cell counts in BALF of *Curvularia* extract and active protease group mice were high along with increased airway narrowing. The mucus secreting cells were also increased in these mice. The changes observed may be attributed to the induction of Th2 type environment with these antigens.

Allergenic proteins can induce activation/degranulation of eosinophils and release of mediators such as eosinophil cationic protein and EPO. The released mediators may induce airway damage and hyper-reactivity in airways (Eum et al., 1995). *Curvularia* extract as well as active protease and ovalbumin induced substantial EPO activity in the present study. The increase in EPO activity of active protease challenged mice may be due to the direct proteolytic activity resulting in mediator release from eosinophils. This was further confirmed as the inactive protease challenged group showed least EPO activity among different antigens tested.

Studies show that proteases from mites and molds react with cell surface receptors in the airways to generate leukocyte infiltration and amplify the immune response to allergens. Such protease activated receptors (PAR) are widely distributed on the cells of airways viz. epithelial cells, mast cells, eosinophils, neutrophils, lymphocyte, smooth muscle, endothelium, fibroblasts and contribute to inflammation. Stimulation of these opens up tight junctions, causes desquamation thereby allowing infiltration of eosinophils. Among these receptors, PAR-2 plays an important role in regulating bronchial eosinophilic inflammation as the effect is abrogated in knockout mice lacking PAR-2. Kauffman has reviewed the effect of airborne protease allergens on the respiratory mucosa (Kauffman, 2003). It was proposed that proallergic 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 p 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 (Sun et al., 2001). PAR-2 dependent pathways have been proposed for chemokines production in protease laden fungal extract induced epithelial cells (Kauffman, 2003). Expression of PARs has also
been reported to influence airway hyperreactivity (Reed and Kita, 2004). In the present study, Cur l 1 sensitized mice showed enhanced allergic response and airway hyperreactivity. Though the exact mechanism by which Cur l 1 augments allergic response is not clear, activation of PAR is one of the probable mechanisms.

The levels of endogenous proteases in body are tightly regulated. Disregulation in levels of endogenous proteases, matrix metalloproteases (MMP-9) and neutrophilic elastase (NE) was correlated with occurrence of asthma (Simpson et al., 2005). Exposure to external proteases probably triggers inflammatory response by a mechanism similar to that triggered by endogenous proteases. Exogenous proteases however can escape the regulatory mechanism. A study suggests that allergic response specific to corresponding proteases (cysteine protease, serine protease, aspartic protease and metallo-protease) was reduced by protease inhibitors (Suzuki et al., 2006). Thus, exposure to protease allergens plays a crucial role in the pathophysiology of allergic respiratory diseases. The observations in the present study show that active protease of Curvularia is more potent in inducing inflammation than inactive protease suggesting proinflammatory effects of protease allergens in airway disease.

Experimental evidences suggest that proteases can facilitate the penetration of other allergens as well. In a study with murine model, limited response was observed when mice were sensitized with purified Asp f 2. The response was heightened when Asp f 2 was administered together with Asp f 13, a purified serine protease. The mice showed exaggerated immune response to both allergens in terms of airway eosinophilia and goblet cell hyperplasia. This validates the role of proteases in mature immune response (Kurup et al., 2002).

In conclusion, the active protease of C. lunata (Cur l 1) is a potent sensitizer than its enzymatically inactive form. The biological activity of protease Cur l 1, though not vital for its allergenicity, contributes significantly towards immune inflammation of airways. The inactive protease may be a better candidate for use in immunotherapy where inflammatory response is not desired.