In recent years, the incidence of asthma and allergies has increased in many countries. As per estimates, 25% of the population worldwide suffers from allergic disorders including asthma (Valenta et al., 1999). The clinical manifestation of allergy is due to cross linking of IgE molecules with specific allergens, bound on the surface of mast cells resulting in mast cell degranulation and release of mediators. Depending on the target organ (tissue), the symptoms are wheezing, cough, breathlessness (lungs), rhinorhea, sneezing (nose), rashes (skin), nausea, vomiting, diarrhea (gastrointestinal tract) and anaphylaxis (systemic reaction). During the first exposure to allergen the sensitization is initiated, while in second or subsequent exposures allergic reaction manifest. The allergens are derived from diverse sources such as fungi, pollens, mites, insects, foods and animal epithelia. Amongst these, the fungi are predominant sources of allergens responsible for type I and type III hypersensitivity disorders.

Aerobiological and clinical studies have revealed that the fungi namely 
*Alternaria alternata*, *Aspergillus fumigatus*, *A. oryzae*, *A. flavus*, *A. niger*, *Cladosporium herbarum*, *Curvularia lunata*, *Epicoccum purpurascens* and *Fusarium solani* are prevalent worldover including India and play a major role in inducing IgE mediated allergic disorders (Bisht et al., 2003; Simon-Nobbe et al., 2008). Some of these fungi like *A. fumigatus* are demonstrated to be pathogenic both in immunocompetent and immunocompromised individuals (Kishor et al., 2002).

Allergen avoidance is the most effective strategy for management of allergies, which may not be possible with a majority of inhaled allergens. The other approach is to make the individual tolerant to the allergen by gradual administration of increasing doses of the allergen. But the individuals may get anaphylactic reactions by administration of crude allergen preparations containing mixture of proteins and other components. Besides, such allergen preparations may develop new sensitization(s) in subjects to irrelevant proteins present in the crude extracts. This limits the scope of current form of immunotherapy with crude extracts for treatment of allergy disorders. Studies suggest that allergen specific immunotherapy using the homogenous preparation of purified allergens is a suitable alternative for treatment of IgE mediated diseases such as allergic rhinitis and/or asthma (Jeong et al., 2006).
The advent of recombinant allergens has led to the development of novel immunotherapeutics for treatment of allergy disorders. The recombinant allergens are homogeneous preparations and offer scope for component resolved diagnosis and treatment. They can be cloned in expression vector of desired trait that aids in easier purification of the allergen as a fusion protein with higher yields (Singh et al., 2006). Further, the recombinant allergens developed have shown similar allergenic potency to that of native counterpart (Best et al., 2000; Shankar et al., 2006).

Various softwares such as DNASTAR, GENE RUNNER, BIOEDIT and INSIGHT are available for homology search, alignment studies, open reading frame search, analysis of restriction enzyme, homology modeling and epitope mapping of allergenic proteins. Study on three dimensional structure of proteins is now possible by computational method using software like PROTEAN. This software enables epitope mapping, and can decipher the various aspects of protein by sequence analysis i.e. percentage amino acid composition, secondary structure, net charge etc. Previously studies were carried out to identify the amino acids essential for IgE binding (Cai et al., 2002). The hypoallergenic variants were obtained using site directed mutagenesis (Swoboda et al., 2002; Shankar et al., 2009) that could bypass IgE cross linking. The knowledge of three dimensional structure of the protein helps in elucidating loops projecting towards the outer aqueous phase and identifying the B cell epitopes. However, the complete determination of three dimensional structure requires the crystallization of the protein. The 3D structure of protein can also be modeled using software with reasonable amount of accuracy for certain purposes. Researchers in this field could develop such software that can model the structure of the protein on the basis of the homology with sequences whose 3D structure has been solved by X ray crystallography. The software’s like INSIGHT can be used for this purpose.

During last 3 decades many allergenic proteins have been identified from diverse sources using standard immuno-biochemical methods. Some of these proteins such as 67, 65, 45, 31 and 26 kDa have been purified and characterized from pollens and fungi (Verma et al., 1998, 2000; Gupta et al., 2004; Shankar et al., 2005). However, it is cumbersome to generate bulk amount of native proteins for molecular
characterization and clinical use. In addition, purified proteins are required to study structure function relationship and mechanism of allergy at the molecular level.

Enzyme (protease) as allergen was first reported in workers of detergent industry by Flindt (Flindt, 1969). In the last decade few other enzyme allergens have been reported and their 3D structure and mechanism of action was predicted (Pasquato et al., 2006; Gonzalez et al., 2002). Enzyme allergens explored in most of the studies are proteases, transferases, enolases, dehydrogenases and chitinases. However, enzyme allergens need to be elucidated for mechanism of action in the target tissue. They are required to be developed in bulk amounts using recombinant DNA technology for *in vitro* and *in vivo* studies in airway disease. The enzyme like glutathione-S-transferase (GST) has antioxidant property but its allergenic nature restricts its use for therapeutic purpose. The variant of this enzyme may be of therapeutic benefit in airway inflammatory disorders.

The present study has been aimed to achieve the following objectives:

**Objectives**

1. To study antioxidant activity of enzyme allergen GST and mutated GST (mGST) in mouse model of airway disease.
2. To evaluate the therapeutic potential of enzymatic and non-enzymatic antioxidants in airway inflammation.
3. To investigate proinflammatory effect of alkaline serine protease (Cur l 1) in mouse model of airway inflammation.
4. Cloning, expression and characterization of a serine protease allergen of *Curvularia lunata*.

**1. Antioxidant activity of GST and mutated GST**

Oxidative stress is an important factor in the pathogenesis of asthma. Furthermore, antioxidant like GST is reduced in asthma patients. In the present study, antioxidant effect of exogenous GST and mGST was investigated in mouse model. Recombinant glutathione-S-transferase was generated as described elsewhere (Shankar et al., 2006). GST was mutated at residues 21/27 to reduced IgE binding. GST and mGST proteins have similar enzyme activity and they were recognized by *A. alternata*-sensitive patients' pooled sera. To evaluate the
antioxidant activity of GST and mGST, BALB/c mice were immunized on day 1 and 14 (10 µg OVA with 1 mg alum) and challenged on days 28, 29 and 30 (4 µg OVA/mice). Mice were given GST, mGST and α-lipoic acid after 1h of each challenge by inhalation in separate groups. The mice were sacrificed on day 31 to evaluate oxidative stress (TBARS in BALF and lung tissue) and inflammatory markers (serum immunoglobulins, BALF cytokines and cellular infiltration).

Oxidative stress as per the lipid peroxidation level in BALF of mGST and GST treated mice was reduced significantly in comparison to PBS treated mice. Mice treated with mGST showed significantly reduced total cell count (p<0.01) and eosinophils (p<0.01) in BALF compared to GST or PBS treated groups. Lung inflammation score was lowest for α-lipoic acid (standard antioxidant) group followed by mGST treated group mice. IL-4 levels were significantly (p<0.01) reduced alongwith OVA specific IgE in mGST group than other treatment groups including GST.

In conclusion, inhalation of mutated GST with reduced allergenicity has better therapeutic potential for attenuating oxidative stress and airway inflammation than GST.

2. Enzymatic and non-enzymatic antioxidants in airway inflammation

Oxidative stress is implicated in the pathogenesis of asthma. Studies show that antioxidant levels are reduced in asthma patients. In our previous study (Chapter 2), mutated GST with reduced IgE binding showed better antioxidant effect and suppressed Th2 response than GST in mouse model. However, the antioxidant effect of mGST was nearly 70% to that of α-lipoic acid, a standard antioxidant. Studies suggest GST (enzymatic antioxidant) catalyses the quenching of reactive oxygen species (ROS) by GSH (non enzymatic antioxidant), and absence of anyone of them may limit antioxidant effect in the system (Rahman et al., 1999). The present study (chapter 3) evaluates the effect of mGST, in combination with GSH, in limiting oxidative stress and Th2 responses in allergen induced mouse model. For comparison, other antioxidants namely mGST, GSH, α-tocopherol and α-lipoic acid were tested individually or in combination
BALB/c mice were immunized on day 1 and 14 (10 µg OVA with 1 mg alum) and challenged on days 28, 29 and 30 (4 µg OVA/mice). Mice were given mGST, GSH, α-lipoic acid, mGST+α-lipoic acid, mGST+α-tocopherol, GSH + α-lipoic acid, GSH+α-tocopherol or mGST+GSH after 1h of each challenge by inhalation and sacrificed on day 31 to evaluate oxidative stress (TBARS and 8-isoprostanes in BALF) and inflammatory markers (serum immunoglobulins, BALF cytokines, NF-kB levels in lung tissue and cellular infiltration).

Mice treated with mGST in combination with GSH showed significantly reduced total cell count (p<0.01) and eosinophils (p<0.01) in BALF compared to other treatment groups including α-lipoic acid. Lung inflammation score in terms of eosinophil infiltration was lowest along with significantly reduced IL-4 for mGST and GSH combination treated group (p<0.01). IL-4 and lung inflammation score in mGST+GSH treated group was comparable to α-lipoic acid, a standard antioxidant. OVA specific IgE reduced maximum in mGST+GSH group than other treatment groups. Oxidative stress as per the lipid peroxidation and 8-isoprostanes level in BALF of mGST with GSH treated mice reduced significantly in comparison to PBS or other antioxidant treatment groups. OVA challenged and PBS treated mice showed significantly raised NF-kB (p65) levels in lung nuclear extract than PBS control. Administration of mGST+GSH combination reduced NF-kB (p65) levels effectively than other treatment groups in mice model.

In conclusion, mGST in combination with GSH has synergistic effect in reducing oxidative stress and airway inflammation. The formulation of mGST+GSH may be explored as an adjunct therapy for asthma management.

3. **Proinflammatory effect of alkaline serine protease**

Studies with mite allergens demonstrated that proteolytic activity augments allergic airway inflammation. But this knowledge is limited to few enzyme allergens. In the present study (chapter 4), we have investigated the effect of serine protease (Cur l 1) allergen of *Curvularia lunata* in airway hyperresponsiveness in allergen induced mouse model.

Cur l 1 was purified and identified as serine protease following protocol inhibitor as described previously (Gupta et al., 2004). Cur l 1, alkaline serine protease was inactivated using specific serine protease inhibitor namely phenyl methyl
sulphonyl fluoride (PMSF). Balb/c mice were sensitized on day 0 with enzymatically active Cur l 1 or C. lunata extract in separate groups. Sensitized mice were given booster dose on day 14 with enzymatically active or inactive Cur l 1. Intranasal challenge was given on day 28, 29 and 30 with separate antigen(s). Airway hyperresponsiveness was measured by plethysmography. The mice were sacrificed on day 31 and blood, bronchoalveolar lavage fluid (BALF), spleen and lungs were collected for analysis of cellular infiltration, immunoglobulins and cytokines.

The enzymatically inactive protein (Cur l 1) has shown >87% inhibition of protease activity. 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 airway hyperresponsiveness (AHR) as compared to OVA, active protease or Curvularia extract group mice (p<0.01). Mice challenged with enzymatically active Cur l 1 demonstrated significantly higher airway inflammation in terms of cellular infiltration than inactive Cur l 1 challenged mice (p<0.01). Serum IgE and IgG1 levels were significantly elevated in mice challenged with active Cur l 1 than inactive Cur l 1 (p<0.01). IL-4 and IL-5 were higher in BALF and splenocyte culture supernatant of active Cur l 1 than inactive Cur l 1 challenged mice. Lung histology also revealed increased eosinophil infiltration in enzymatically active Cur l 1 group mice. Lung sections from OVA/Curvularia extract/active protease group mice showed narrowed airway lumens with enlarged epithelial cells and mucus plugs. The inactive protease group mice showed lesser goblet cell hyperplasia in lungs than active protease challenge.

The results suggest that proteolytic activity of Cur l 1 plays an important role in enhancing airway inflammation in mice. The enzymatically inactive Cur l 1 may be explored for immunotherapy of fungal allergy.

4. Cloning, expression and characterization of new allergen of C. lunata

Recombinant allergens are useful tools for component resolved diagnosis and therapy of allergic disorders. In addition, recombinant proteins are required to decipher mechanism of allergy at molecular level. In the present study (chapter 5),
serine protease a major allergen from *Curvularia lunata* was cloned, expressed, purified and analyzed for its immunological reactivity.

cDNA library was screened with sera obtained from *C. lunata* allergic patients. Among different allergenic clones screened, one of the clone showed homology to subtilisin like serine proteases from different fungal sources. Full length clone was generated by RACE PCR (Rapid amplification of cDNA ends - polymerase chain reaction). ORF (open reading frame) of the gene was identified and in silico translated protein showed homology with serine proteases from different sources like *Pyrenophora tritici,* *Penicillium oxalicum,* *P. citrinum,* *A. fumigatus,* *A. niger* etc. The gene was was cloned in pET22b+, expressed in *E. coli,* purified from inclusion bodies and characterized for allergenicity by immuno-biochemical methods.

Full length cDNA clone was 1521bp. In-silico translation of the sequence has given a protein of 506 amino acids. NCBI-BLAST search has shown conserved domains for subtilisin like serine protease. After cloning and expression, the protein was purified to homogeneity using Ni-NTA column. It resolved at 54-kDa as a single band on 12% SDS-PAGE. The protein transferred to nitrocellulose membrane reacted with *Curvularia* sensitive patients’ pooled sera. The protein has recognized polyclonal antibodies against serine proteases from *C. lunata* and *Periplaneta americana* indicating it to be a serine protease. Mass spectrometric analysis of purified protein has shown *Candida albicans,* *Penicillium chrysogenum* and *Aspergillus nidulans* as its closest homologs in matrix science database. The recombinant protein was recognized by 13 of 16 (> 80 %) *Curvularia* positive patients’ sera in ELISA and immunoblot demonstrating it to be a major allergen of *C. lunata*. To check the specificity and potency of IgE binding ELISA inhibition was performed with pooled patients’ sera. The purified protein showed dose dependent inhibition using self protein as inhibitor. It required 50-56 ng of self protein for 50% inhibition of IgE binding (EC50) on solid phase coated 54 kDa protein. Whereas, for 50% inhibition of solid phase bound *C. lunata* 172 ng of purified protein was required.

In conclusion, recombinant 54-kDa major allergen of *C. lunata* was cloned, expressed, and characterized by standard bioinformatic and immunological
methods. The protein has high biological potency and diagnostic efficiency of 81% for *C. lunata* sensitive patients.

**CONCLUSIONS**

- Allergic activity of enzyme allergen glutathione-S-transferase (GST) restricts its therapeutic use as an antioxidant in airway disorders.

- GST mutated at amino acid positions 21 and 27 (mGST) has shown reduced IgE binding and retained enzymatic activity.

- Administration of mGST intranasally reduced oxidative stress and Th2 response in mouse model of allergy.

- Enzymatic antioxidants like GST or mGST show limited therapeutic effect in absence of complementary non enzymatic substrates.

- mGST (enzymatic antioxidant) in combination with GSH (non enzymatic substrate) shows better antioxidant activity as compared to individual antioxidants.

- mGST+GSH combination shows better therapeutic effect than other antioxidant(s) individually or in combination for ameliorating inflammatory markers in allergen induced mouse model.

- mGST+GSH formulation gives comparable therapeutic effect in reducing oxidative stress and airway inflammation to that of α-lipoic acid, the standard antioxidant in mouse model.

- Protease activity of Cur l 1, a major allergen of *C. lunata* plays an important role in progression of airway inflammation in mouse model.

- Airway challenge with inactive Cur l 1 in antigen sensitized mice shows reduced inflammation than enzymatically active counterpart.

- Inactive Cur l 1 may be explored for immunotherapy of fungal allergies.
Summary and Conclusions

- A 54 kDa protein was cloned, expressed, purified and characterized for its allergenicity.
- The 54 kDa protein has been identified as major allergen of *C. lunata* as it reacted with 13 of 16 individual patients’ sera.
- The protein reacted with anti-Cur l 1 (alkaline serine protease from *C. lunata*) demonstrating it to be a serine protease and homology search further categorized it as subtilisin like serine protease.
- Being a potent major allergen, the subtilisin like protease (54 kDa) can be explored for component-based diagnosis and therapy of fungal allergy.