Summary & Conclusions
A large number of food allergens, usually proteins capable of inducing allergic symptoms, including severe, even life-threatening reactions in predisposed individuals, have been identified and characterized. As most of these proteins are from our daily dietary intake, they are often difficult to avoid. Every protein is not allergenic but, there is certain protein that possess IgE binding site known as epitope. The epitope containing proteins are known as allergens. Detailed knowledge about the characteristics of food allergens, their structures, biological activity, and stability, may be helpful in improving diagnosis of food allergy, avoiding unnecessary exclusion of diets, and assessing the risk of cross-reactive allergies to other food sources Red kidney bean (*Phaseolus vulgaris* L.), is a common legume, consumed worldwide. The delicacy of red kidney bean is highly appreciable but, at the same time their toxicity and allergenicity have raised alarming concern. Hence, following studies are proposed as a part of this dissertation to fill up the above mentioned lacunae in RKB induced allergenicity:

- Prevalance of red kidney bean (*Phaseolus vulgaris* L.) allergy in human subjects: Identification of pepsin resistant and IgE binding proteins.
- Allergenic responses of red kidney bean (*Phaseolus vulgaris* L.) crude protein extract in BALB/c mice.
- Partial characterization of IgE binding proteins of Red kidney bean (*Phaseolus vulgaris* L.) using proteomic and bioinformatic approaches.
- Purification, characterization and allergenicity assessment of phaseolin.
Prevalence of red kidney bean (Phaseolus vulgaris L.) allergy in human subjects: Identification of pepsin resistant and IgE binding proteins

Out of 350 allergic patients, 20 (5.7%) patients showed positive results to SPT, elevated levels of total IgE, specific IgE and IgG1. These patients showing susceptibility toward RKB were suffering from allergic rhinitis, nasobronchial asthma, urticaria and dermatitis. Considering the immediate onset 28.6% of the RKB allergic patients (n=6) had claimed symptoms of abdominal pain and cramps, indigestion and diarrhea on ingestion of RKB dishes. Twenty allergic patients showed positive SPT results (+1 to +3) against RKB allergen extract. Serum total IgE and specific IgE level ranged between 196 and 663 kU/mL and 1.74 and 11.9 kAU/L respectively, while in normal healthy volunteers, it ranged between 39.5 to 232 kU/L and 0.27–0.55 kAU/L, respectively. Enhanced levels of IgG1 (OD 1.0–3.6) were observed in the RKB SPT+Ve patients over control (OD 0.05–0.6). Interestingly, RKB was found to show cross-reactivity to peanut, soybean, chickpea and Bengal gram. The serum samples preincubated with peanut, chickpea, soybean and black gram exhibited a significantly reduced antibody binding to RKB extracts in a dose dependent manner. ELISA inhibition test with peanut, chickpea, soybean, and black gram have showed inhibition up to 74%, 74%, 55% and 71% at 100 µg/mL.

The protein profile of RKB-CPE showed a wide range of proteins with molecular weight 170 to 10 kda. The proteins with molecular weight 43-50 and 34 kDa were found in the majority. Immunoblotting with pooled sera of RKB allergic patients showed five IgE reactive proteins of molecular weight approximately 170, 100,
43-50, 34 and 20-25 kDa. The strip incubated with normal human and control mice sera did not show any band.

No significant change has been observed in the levels of major proteins of RKB-CPE. Further, densitometry study of three major proteins (100, 43-50 and 34 kDa) of RKB-CPE showed 61, 83 and 85% relative density even after 30 minutes incubation at 90°C. Five protein components of molecular weight approximately 170, 100, 50-43, 34 and 20-25 kDa were found to be pepsin resistant. Densitometry analysis revealed that percentage of 170 kDa protein component that remained undigested was 96, 95, 92, 90, 86, 80, 82 and 79% at time periods 0, 0.25, 1, 2, 4, 8, 15 and 60 min in SGF, respectively. Similarly, 100 kDa protein components remaining after SGF digestion was 91, 91, 88, 85, 82, 75, 77, 73% at time period mentioned above. Also, 43-50 kDa protein components the percentage density of undigested protein was 84, 82, 70, 64, 64, 61, 57, 57%. Densitometry analysis of 34 kDa protein component after SGF digestion showed 63, 67, 60, 61, 54, 54, 42, and 37%. Analysis of 20 kDa protein component density of remaining protein was 70, 66, 60, 60, 58, 53, 42 and 39% at time periods 0, 0.25, 1, 2, 4, 8, 15 and 60 min in SGF, respectively.

Five protein components of molecular weight approximately 170, 100, 43-50, 34 and 20-25 kDa were found to be resistant in the SIF. Densitometry analysis revealed that percentage of 170 kDa protein component that remained undigested was 89, 85, 77, 70, 67, 63 and 59% at time period 0, 1, 5, 10, 15, 60 and 120 min in SIF, respectively. Similarly, 100 kDa protein components remaining after SIF digestion was 93, 81, 64, 64, 48, 45 and 39% at time period mentioned above.
Also, 43-50 kDa protein components the percentage density of undigested protein was 96, 95, 80, 81, 69, 59, 44%. Densitometry analysis of 34 kDa protein component after SIF digestion showed 93, 88, 85, 89, 78, 73 and 72%. Analysis of 20 kDa protein component density of remaining protein was 80, 79, 78, 88, 95, 100, and 109% at different incubation time periods.

**Allergenic responses of red kidney bean (Phaseolus vulgaris L.) crude protein extract in BALB/c mice**

The level of total IgE, specific IgE and IgG1 were significantly increased in 15, 43 and 59 days in the serum of RKB-CPE treated mice. The IgE binding potential of RKB-CPE was also confirmed by the pooled sera of RKB treated mice which indicated five IgE binding proteins with approx molecular weight 170, 100, 43-50, 34 and 20-25 kDa. The mice sensitized and challenged with RKB-CPE showed scratching around the nose and mouth (anaphylactic score 2; 20%), forced respiration (score 3, 60%); and 20% showed symptoms of score 4, like, severe diarrhea including unconsciousness or negligible response despite the gentle touch. A decrease of 3, 2.5 and 2°C in the rectal temperature of red kidney bean sensitized and challenged mice was noticed after 40 min of challenge in 3 (60%) mice, 1 (20%) mice and 1 (20%) while the control mice failed to show any decrease in the rectal temperature. The severity of anaphylaxis induced post red kidney proteins challenge was found to be augmented in form of plasma histamine and serum mMCP1 in treated and challenged mice. An enhanced level of the peritoneal albumin level was observed in the serum of red kidney
proteins treated and challenged mice. Further, increase in the serum TSLP level was observed in RKB treated mice.

Histopathology of the lungs of RKB-CPE treated mice showed thickening in the alveolar septa and mucus infiltrations. Exfoliations in the intestinal walls and leukocytes infiltrations were evident in the intestine of RKB-CPE treated mice. The spleen of RKB-CPE treated mice showed the presence of megakaryocytic structures. No such changes were evident in control groups.

A significant release of β-hexosaminidase was observed following exposure of different doses of RKB-CPE in RBL-2H3 cells. The percentage release was 58.96, 64.22, 68.24, 72.16, 75.36, and 74.57% in 20, 40, 60, 80, 100 and 120 μg RKB-CPE. Further, the significantly enhanced mean intensity of GATA-3, STAT-6, c-MAF, T-bet and NFATc1 were found in RKB-CPE treated group.

The allergenicity of RKB was evident by significantly enhanced levels of total IgE and specific IgE, prominent anaphylaxis symptoms, reduced core body temperature, higher plasma histamine and mMCP-T1 levels. The eosinophil counts and MPO level in RKB-CPE treated mice. Increased mast cell counts were evident in the lungs; intestine and spleen of RKB challenged mice. RKB-CPE treated mice showed a positive reaction in the type 1 skin test in sensitized mice. Significantly enhanced mean intensity of GATA-3 and T-bet was obtained in the RKB CPE group. We next sought to determine the ex vivo responses of RKB-CPE on splenocytes of RKB sensitized vs. untreated groups. The RKB-U and RKB-T splenocytes secreted enhanced levels of IL-1β, IL-2, IL-4, IL-5, IL-12 and IL-13.
After analyzing cytokines, we tried to explore the role of transcription factors involved in the regulation of allergic manifestations induced by RKB-CPE in splenocytes. Expression of GATA-3 was found to enhance in RKB-T group as well as RKB-U group. The STAT-6 level was elevated in both RKB-U and RKB-T group over the control. The T-bet and c-MAF expressions were found elevated in the RKB-U group but not in RKB-T group. An increase of 8% in CD4+T cells and 1% decrease in CD8+T cell population was observed in RKB group when compared to control. The B-cells population in RKB exposed group was increased by 11.6% over the control.

We next sought to reveal the release of allergic mediators in the in vitro and in vivo conditions following RKB CPE exposure. The BMMC, PCMC and RBL-2H3 cells showed a significant enhancement in the release of β-hexosaminidase following RKB-CPE (at 100 and 125 µg in all groups) treatment over control. The increased levels of histamine and CysL got elevated by 5 and 6 fold, respectively in RKB-CPE treated group when compared to control in RBL-2H3 cells.

The specific hemagglutination activity of RKB, RKB-A and PHA-P was 204, 0.2 and 204 titer/mg. We further extended our study in female BALB/c mice to quantify specific IgE and IgG1 levels induced by PHA-P treatment. The level of specific IgE was found significantly enhanced in RKB, RKB-A and PHA-P, respectively when compared to that of control on day 15th, 43rd and 59th. The level of specific IgG1 was found significantly enhanced in RKB, RKB-A and PHA-P treated groups in comparison to control.
The mice from different PHA-P treated group exhibited symptoms of systemic anaphylaxis 40 min after challenge. Mice in the PHA-P treated and challenged group exhibited scratching and rubbing around the head and snout (score 1) in 10% mice; pilar erection, puffiness around the eyes and snout, reduced activity or standing still, increased respiratory rate and diarrhea (score 2) in 20% mice; symptoms of score 1 and score 2 along with labored respiration (score 3) was shown in 30%; near fatal reactions such as loss of consciousness or little activity despite gentle prodding (score 4) was evident by 30% mice. Mortality (score 5) was noted in 10% mice. In this study, 8 out of 10, PHA-P treated and challenged mice showed 3 to 4°C decrease in core body temperature.

A 2 fold increase in the plasma histamine level was observed in PHA-P treated mice when compared to control. In our result, PHA-P sensitized mice showed 2.5 fold increases in the concentration of TSLP in comparison to control. More than 2 fold enhancements in mMCPT-1 were observed in the serum of mice treated with PHA-P when compared to control.

We further extended this study using RBL-2H3 as in vitro model and assessed mediator release after exposure of RKB, RKB-A and PHA-P. The β-hexosaminidase levels were found elevated at all concentration of RKB, RKB-A and PHA-P. Further, PHA-P treated group indicated up to 3 fold enhanced level of β-hexosaminidase release at 25, 50, 75, 100 and 125 µg concentrations when compared to control. The level of histamine, PGD₂, and CysL were found to be enhanced in RKB, RKB-A and PHA-P treated RBL-2H3 cells. In the PHA-P
treated RBL-2H3 cells, the level of histamine (1.7 fold), PGD₂ (5 fold) and CysL (4 fold) were found enhanced when compared to their respective control.

**Partial characterization of IgE binding proteins of red kidney bean** (*Phaseolus vulgaris* L.) **using proteomic and bioinformatic approaches**

The IgE binding RKB proteins with approximate Mol wt 130 kDa, 70 kDa, 43-50 kDa, 34 kDa and 20-25 kDa were recognized by individual serum of RKB SPT+Ve patients. The sera of healthy volunteers did not show any reactivity to RKB proteins. The proteins with Mol. wt. 43-50 kDa and 34 kDa were evident in the majority of RKB allergy patients.

One and 2D SDS-PAGE of RKB-CPE showed the presence of a wide range (molecular weight 170-10 kDa) of proteins with isoelectric point (pI) range 4 to 7. Further, 2D IgE immunoblotting using red kidney bean allergic patients’ sera recognized a total of twelve IgE-binding proteins (IgE BP-1 to 12) in red kidney bean. On the basis of the MOlecular Weight Search (MOWSE) score obtained after LC-MS/MS analysis, five IgE-BP of red kidney bean crude protein extract (IgE BP-2, IgE BP-4, IgE BP-8, IgE BP-10 and IgE BP-12) were characterized as legumin, phaseolin, IAA protein conjugate, albumin-2 and phytohemaglutinin.

Bioinformatics analysis according to SDAP tool showed similarity of legumin, phaseolin, IAA-protein conjugate, albumin-2 and phytohemagglutinin with other known food allergens including Gly m 6.0401 of soybean (*Glycine max*), Vig r 2.0101 of green gram (*Vigna radiata*), Per v 1 of tropical green mussel (*Perna*)
viridis), Vig r 4.0101 of green gram (Vigna radiata), Gly m lectin of (Glycine max) etc.

The allergenicity of legumin was predicted on the basis of SVM module based on amino acid composition, BLAST search on ARPs and hybrid approach. Algpred results also indicated phaseolin can have allergenic properties on the basis of mapping of IgE epitopes and PID, SVM module based on amino acid and dipeptide compositions, BLAST search on ARPs and hybrid approach. The phaseolin has sequences SYLQEFSKHI and SYLQEFSKHI at the position 180, respectively which is very similar to IgE epitope SYLQEFSRN. The IAA-protein conjugate was also predicted allergic protein, but only on the basis of SVM module based on dipeptide composition. Algpred results indicates albumin-2 can be an allergen too on the basis of SVM module based on amino acid composition, SVM module based on dipeptide composition, and hybrid approach. The full FASTA, sliding 80mer and 8mer exact matches showed that legumin, phaseolin and phytohemagglutinin may have allergenic properties while no match was found in the case of IAA-conjugate proteins and albumin-2.

**Purification, characterization and allergenicity assessment of phaseolin**

The 80-95% ammonium sulphate fraction showed 43-50 kDa protein of RKB in majority. Further, fractions 22-25 of HiTrap column showed single bands of mol weight 43-50 kda with some impurities. The SDS-PAGE of RKB-CPE showed the presence of a wide range (170-10 kDa) of proteins. The protein of approximately Mol. wt. 50 kDa protein appeared in the unbound fraction as a single band on
12% SDS-PAGE and a single peak at a retention time 6.493 min on RP-HPLC. The PMF studies suggested the purified protein as phaseolin with molecular weight 47.53. The phaseolin showed resistant to pepsin up to 60 min in the SGF. The percentage density of phaseolin were 99.0, 91.6, 96.5, 81.5, 75.0, 73.0, 69.5, 65.7 and 56.5% at the incubation time 0, 0.25, 0.5, 1, 2, 4, 8, 15 and 60 min, respectively. The purified allergen was recognized by 75% (15/20) of the individual sera from patients with RKB allergy. Individual healthy volunteer’s serum did not so any binding with phaseolin. The RKB allergy patients showed increased specific IgE against phaseolin when compared to that of healthy volunteers. Sera of phaseolin sensitized mice also showed the IgE binding with phaseolin while control mice sera did not so any such binding.

Bioinformatics analysis using AllergenOnline tools confirmed the possibility of phaseolin allergenicity. The 8 mer (N= 414; Number of sequences with at least one 8mer match = 10) study revealed the similarity of phaseolin with the beta-conglycinin storage protein (*Glycine max*), Ara h 1 allergen (*Arachis hypogaea*) etc. The 80mer (N= 342; Number of Sequences with hits 32) study revealed the similarity of phaseolin with CG4 β-conglycinin (*Glycine max*), allergen Len c 1.0102 allergen and Len c 1.0101 (*Lens culinaris*), Vicilin (*Pisum sativum*), conarachin (*Arachis hypogaea*), conglutin beta (*Lupinus angustifolius*), vicilin seed storage pr (*Juglans nigra*) and 7S globulin (*Sesamum indicum*).

Further, Algpred results also indicated that phaseolin can be an allergenic protein on the basis of mapping of IgE epitopes and PID, SVM module based on amino acid and dipeptide compositions, blast search (Hits found in ARPs database:
PHFNSKAMVIVVVKGTGNLELVA) on allergen representative peptides (ARPs) and hybrid approach (SVMc+IgE epitope+ARPs BLAST+MAST). The phaseolin has sequences SYLQEFSKHI at the position 180 which is very similar to IgE epitope SYLQEFSRNT.

The search was also performed in the SDAP allergen database for allergenicity of phaseolin. The exact match for contiguous amino acids (n=6), showed that phaseolin share similarity with known allergens like Vig r 2.0101, Lol p 11.0101, Phl p 11.0101, Gly m 5.0101, Gly m conglycinin, etc. FASTA alignments for an 80 amino acids sliding window showed the similarity of phaseolin with Vig r 2.0101, Gly m conglycinin, Gly m 5.0201, Vig r 2.0201 etc. Enhanced levels of sIgE, sIgG1, MCP-1, prominent anaphylaxis symptoms, reduced core body temperature and mMCP-1 levels were evident in the phaseolin treated mice when compared to control. The intestine of phaseolin treated mice showed mild goblet cell hyperplasia, exfoliation in the intestinal wall, the spleen showed lymphoid hyperplasia, megakaryocytes and edema. The lungs showed congestion, thickening in the alveolar septa and edema. A positive reaction to type I skin test was evident in the phaseolin challenged mice when compared to control. Phaseolin challenged group showed increase in the level of mast cells in the lungs, spleen and intestine when compared to the respective controls.

The mRNA levels of *IL-4*, *IL-5* and *IL-13* were found up-regulated in the lungs, spleen and intestine of phaseolin sensitized mice when compared to that of controls. The expression of *GATA-3* was found up-regulated in the intestine while
there was no significant change in the expressions of foxp3 and T-bet in comparison to controls.

The levels of GATA-3 were found significantly enhanced in the lungs, spleen and intestine of phaseolin treated mice when compared to that of control mice. We next sought to determine the \textit{ex vivo} responses of phaseolin on splenocytes. The phaseolin treated splenocytes secreted enhanced levels of IL-4, IL-5 and IL-13 when compared to respective control. The BMMC showed 43, 44, 47, 65 and 59% release of β-hexosaminidase following exposure of 20, 40, 60, 80 and 100 µg phaseolin treatment over control.

Taken together, the findings of the present dissertation can be summarized as:

1. The prevalence of RKB allergy was evident in 5.7% allergic patients. These patients showed clinical history of nasobronchial asthma, allergic rhinitis, dermatitis and urticaria.

2. The levels of total IgE, specific IgE and IgG1 were found elevated. The IgE immunoblotting using RKB allergic patient’s sera showed five IgE binding proteins in RKB of approx molecular weight 170, 100, 43-50, 34 and 20-25 kDa.

3. The simulate gastric fluid (SGF) and simulate intestinal fluid (SIF) assays showed five pepsin resistant proteins with approx molecular weight 170, 100, 43-50, 34 and 20-25 kDa.

4. RKB-CPE treated mice showed enhanced levels of total and specific IgE, anaphylactic symptoms, histamine, and mouse mast cell protease-1 (mMCPT-1).
5. An enhanced release of β-hexosaminidase release was observed in the passively sensitized RBL-2H3 cells exposed with RKB-CPE.

6. Based on enhanced levels of specific IgE, anaphylaxis score, histamine and abnormal histopatholy, it can be concluded that PHA-P may augment RKB induced allergenicity.

7. One dimentional IgE-immunoblotting using individual patient’s serum demonstrated IgE binding proteins with approx molecular weight 170, 100, 43-50, 34 and 20-25 kDa in RKB-CPE.

8. Twelve RKB-CPE proteins showed IgE binding capacity in two dimensional IgE-immunoblotting using pooled sera of RKB allergic patients.

9. Five IgE-binding proteins were characterized using LC-MS/MS as legumin, phaseolin, IAA-protein conjugate, albumin-2 and phytohemagglutin.

10. Phaseolin was purified by ammonium sulphate fractionation, anion exchange and gel exclusion chromatography and characterized by peptide mass fingerprinting (PMF) as a major IgE binding protein of RKB.

11. Phaseolin treated mice showed enhanced levels of specific IgE and IgG1, monocyte chemtactic protein (MCP-1), anaphylaxis symptoms, histopathological changes, mRNA expressions of IL-4, IL-5, IL-13 and GATA-3 in the lung, spleen and intestine and IL-4, IL-5 and IL-13 in phaseolin exposed splenocytes culture supernatants over control.