Chapter 5
Chapter 5
Evaluation of allergenic potential, identification & characterization of green gram allergens

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5.1. Introduction

Green gram (*Vigna radiata*), a native of India belongs to family Leguminoseae and commonly known as ‘mung bean’. Green gram is one of the major legumes, as it is widely cultivated throughout Asia, including Pakistan, Bangladesh, Sri Lanka, Thailand, Laos, Cambodia, Vietnam, Indonesia, Malaysia, China, Formosa and Texas as well as California states of U.S.

Green gram contains about 25 percent protein, which is almost 2.5 times that of cereals (http://agmarknet.nic.in/Greengram_profile.pdf). It supplies protein requirement of vegetarian population. Green gram is consumed in the form of whole as well as split pulse, and supplement cereal based diet. It is commonly used for sprouting in China, India and Western countries. The mung bean ‘Khichdi’ is recommended to the ill or aged persons as it is believed that it is easily digestible and considered as a complete diet. It is particularly rich in leucine, phenylalanine, lysine, valine and isoleucine amino acids. Green gram is also an excellent source of vitamins, minerals and proteins (Mubarak, 2005). Green gram consumption produces small increase in blood glycemic index in humans making it an attractive option for diabetic patients. It has also been reported to modify glucose and lipid metabolism favorably in rats (Borret et al., 1989; Lerer-Metzger et al., 1996). Antifungal and antibacterial activity (Wang et al., 2004) of certain proteins of green gram is well documented probably because it contains phenolic substances/ α-amylase inhibitors which interact with digestive enzymes thereby modulating their activity (Alarcon-Aguilar et al., 2005). Additionally, ‘mung bean’ sprout was found to inhibit the growth of the bacteria *Helicobacter pylori* that causes gastro duodenal disease thereby showing its antimicrobial potential (Randhir et al., 2004).

Despite its various beneficial properties and high consumption, adverse reactions to green gram have not been investigated probably
because of assumption of its easy digestion. However, we have reported in chapter 3 that green gram caused sensitization in allergic population and ranked second after chickpea in our study performed on naso-bronchial allergic patients. Moreover, a protein from green gram seedling, Vgr 1 had been identified as an allergen (Mittag et al., 2005); however, no work on allergens and resulting allergenicity from green gram seed (major eatable part) has been carried out. Thus, it is looking relevant to study allergenic potential of green gram. The aim of this study was to detect, identify and characterize allergenic proteins of green gram, which could be the cause of allergic reactions. Therefore, present investigation attempts to study the allergenic potential of green gram proteins in BALB/c mice as well as in patients of naso-bronchial allergy. Studies have also been undertaken to identify allergenic proteins of green gram that show resistance against simulated gastric fluid (SGF) and specific IgE binding. Mass spectrometry analysis was undertaken after 2 D electrophoresis in combination to bioinformatics tools to characterize the novel allergenic proteins of green gram.

5.2. Materials and Methods
5.2.1. Chemicals and reagent kits
All the chemicals and reagent kits were similar as described in chapter 4, section 4.2.1. Dry seeds of green gram (Vigna radiata) were purchased from local market.

5.2.2. Reagents
All reagents were prepared as described in chapter 4, section 4.2.2.

5.2.3. Preparation of green gram CPE
Protein from green gram was extracted and protein content of CPE was estimated as described previously in chapter 2, sections 2.2.4 and 2.2.5.
5.2.4. Study Subjects
Nine green gram allergic patients were identified and selected from a population with nasal-bronchial allergy during a prospective study performed at the Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University, Lucknow, India. The selection was carried out on the basis of a convincing clinical history of allergic symptoms after consuming green gram, which was confirmed on the basis of positive SPT. SPT and blood collection were carried out with the consent of patients and the study protocol was approved by the human ethics committee of the institute.

5.2.5. SPT and sera collection
SPT and sera collection was performed as described previously in chapter 3, section 3.2.3. Individual patient's sera were used for detection of IgE binding proteins by immunoblotting. Pooled sera of these 9 subjects were used in ELISA inhibition assays and IgE immunoblotting of 2DE resolved green gram proteins. A pooled serum from other 10 patients allergic to dander and insects but not to plant foods or pollens was used as negative control.

5.2.6. Total IgE estimation
Determination of total IgE levels in patient's serum was performed as described previously in chapter 4, section 4.2.6.

5.2.7. Specific IgE estimation
Specific IgE levels against green gram were estimated by enzyme-linked immunosorbent assay as described previously in chapter 4, section 4.2.7.

5.2.8. ELISA inhibition
The allergenic potency of green gram extracts and its cross-reactivity with other commonly consumed legumes like soybean, red gram, chickpea and
bengal gram was determined *in vitro* by ELISA inhibition as described previously in chapter 4, section 4.2.8.

5.2.9. Animal studies
Details of animal studies are given in chapter 4, section 4.2.9.

5.2.9.1. Animal sensitization protocol
Mice were treated intraperitoneally as described previously in chapter 4, section 4.2.9.1.

5.2.9.2. Blood samples collection
Blood samples were collected as described previously in chapter 4, section 4.2.9.2.

5.2.9.3. Measurement of systemic anaphylactic responses
Anaphylactic signs in sensitized mice were evaluated 30 to 40 minutes after challenge mice (n=5) with 1 ml of 10 mg/ml green gram CPE intraperitoneally on 60th day as described previously in chapter 4, section 4.2.9.3.

5.2.9.4. Determination of plasma histamine levels
Histamine assay was carried-out as described previously in chapter 4, section 4.2.9.4.

5.2.9.5. Histopathological processing
Challenged mice in each group were sacrificed by cervical dislocation on day 60. Lungs, spleen and intestine were taken out for histopathology as described previously in chapter 4, section 4.2.9.5.

5.2.9.6. Total IgE estimation
Determination of total IgE levels in serum was performed as described previously in chapter 4, section 4.2.9.6.
5.2.9.7. Protein specific IgE, IgG1 and IgG2a antibodies estimation
Serum antibodies specific for green gram was measured by indirect ELISA as described previously in chapter 4, section 4.2.9.7.

5.2.9.8. Isolation and culture of splenocytes
Splenocyte culture was performed as described previously in chapter 4, section 4.2.9.8.

5.2.9.9. Cytokine determination
The cytokine content of culture supernatants derived from splenocytes following 24, 48 and 72 h in culture was determined as described previously in chapter 4, section 4.2.9.9.

5.2.9.10. RT-PCR analysis of cytokine m-RNA
A semi-quantitative RT-PCR analysis of Th1 (IL-2, IL-12 and IFN-γ) and Th2 (IL-4, IL-5 and IL-10) cytokines mRNA in blood and spleen from mice sensitized with green gram and peanut CPE was performed as described previously in chapter 4, section 4.2.9.10.

5.2.10. Identification and characterization of green gram allergens
5.2.10.1. In vitro digestibility of green gram CPE
*In vitro* SGF was performed as described by Roesler and Rao (2001) with slight modifications as mentioned in Chapter 2, section 2.2.7. SGF digestion was stopped after 2 min by addition of stopping solution and the resultant products was resolved over 12% SDS-PAGE according to the method of Laemmli (1970) and stained with CBB (G-250).

5.2.10.2. Immunoblotting of green gram CPE
Immunoblotting was performed for detection of specific IgE binding proteins of green gram, using sera of patients allergic to this legume as described.
previously in chapter 4, section 4.2.10.2. Green gram CPE immunoblots were compared with 2 min SGF digests of green gram to see the allergenic potential of pepsin stable proteins.

5.2.10.3. Two-dimensional electrophoresis (2 DE) and immunoblotting of green gram proteins

2DE and immunoblotting of green gram protein extract was carried out as described in chapter 4, section 4.2.10.3.

5.2.10.4. Liquid chromatography mass spectrometry (LC-MS/MS) analysis of IgE immunoreactive green gram proteins

Proteins showing IgE binding with green were subjected to mass spectrometry as described in chapter 4, section 4.2.10.4.

5.2.10.5. Sequence analysis

High probability based MOWSE score were used to identify proteins. To decipher probable protein sequence from tryptic digestion generated peptides, alignment of individual peptide sequence was run with the protein hit having maximum probability score using CLUSTAL W program on Biology Workbench 3.2 (www.workbench.sdsc.edu). The resulting protein sequence was subjected to BLAST search on Gene Bank protein database to confirm the identity of LC/MS-MS results and to know the super families of these identified proteins. Also, FASTA search on ‘www.allergenonline.com’ was carried out to determine allergenicity of proteins.

5.2.11. Statistical analysis

Values are presented as means ± SEM. Statistical evaluation was carried with one-way ANOVA (Snedecor and Cochran, 1967). In all the cases, p values less than 0.05 were considered significant when compared to controls.
5.3. Results

5.3.1. Patients study
Nine selected patients with marked positive SPT (2+ or more) to green gram extracts had total IgE levels, ranging from 706 to 5054 IU/ml. Similarly, patients allergic to dander and insects (negative control) had total IgE levels ranging from 225-4305 IU/ml. Specific IgE levels in green gram allergic patients were more than 3.02 fold elevated over negative control. All the patients elicited marked positive skin reactions to other legumes as evident by 77% to red gram, 66% to soybean, 55% to chickpea, 33% to bengal gram, 10% to lentil and bean fresh each (Table 5.1).

5.3.2. Potency of green gram extract and its cross-reactivity with other legumes
Allergenic potency of the green gram extract was determined by ELISA inhibition. Green gram extract required 76 ng of self protein for 50% inhibition (Fig. 5.1 a). To assess the cross-reactivity of green gram with other legumes (as evident by SPT also), ELISA inhibition was performed using red gram, soybean, chickpea and Bengal gram extracts as inhibitors. These legumes produced 50% inhibition of specific IgE binding to solid phase green gram extract with 320 ng, 360 ng, 400 ng and 480 ng protein, respectively. The maximum inhibition (85%) was observed with red gram followed by 81% with soybean, 75% with bengal gram and 74% with chickpea, at 5 μg protein extracts (Fig. 5.1 b).

5.3.3. Total IgE, specific IgE, IgG1a and IgG2a levels in mice sera
BALB/c mice were sensitized with either green gram or peanut and serological responses were analyzed using indirect ELISA. On days 15, 43 and 59, total IgE levels in both green gram and peanut (positive control) sensitized group were significantly higher (p<0.001) as compared to vehicle
Table 5.1: Clinical and immunologic analysis of green gram-sensitive respiratory allergic patients

<table>
<thead>
<tr>
<th>Patients No.</th>
<th>Age (years)/Sex</th>
<th>Symptoms*</th>
<th>SPT with green gram CPE</th>
<th>Total IgE (IU/ml)</th>
<th>Specific IgE (OD)</th>
<th>Sensitization to other legumes**</th>
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<tbody>
<tr>
<td>1. 33/M</td>
<td>AR/BA</td>
<td>2+</td>
<td>706.39</td>
<td>1.89</td>
<td>Rg, Sb, Bg</td>
<td></td>
</tr>
<tr>
<td>2. 26/M</td>
<td>AR</td>
<td>2+</td>
<td>1288</td>
<td>1.09</td>
<td>Rg, Bn, Ln</td>
<td></td>
</tr>
<tr>
<td>3. 20/M</td>
<td>AR/BA</td>
<td>2+</td>
<td>1245.90</td>
<td>1.29</td>
<td>Rg, Ch</td>
<td></td>
</tr>
<tr>
<td>4. 34/M</td>
<td>AR/URT</td>
<td>2+</td>
<td>2029.09</td>
<td>1.54</td>
<td>Rg, Bg, Ch</td>
<td></td>
</tr>
<tr>
<td>5. 45/F</td>
<td>BA</td>
<td>2+</td>
<td>848</td>
<td>1.33</td>
<td>Sb, Bg</td>
<td></td>
</tr>
<tr>
<td>6. 16/F</td>
<td>BA/GIS</td>
<td>2+</td>
<td>1142</td>
<td>1.39</td>
<td>Sb, Ch</td>
<td></td>
</tr>
<tr>
<td>7. 40/M</td>
<td>AR/BA</td>
<td>2+</td>
<td>1110</td>
<td>1.82</td>
<td>Rg, Ch, Sb</td>
<td></td>
</tr>
<tr>
<td>8. 32/M</td>
<td>AR/BA</td>
<td>2+</td>
<td>725</td>
<td>1.76</td>
<td>Rg, Sb, Ch</td>
<td></td>
</tr>
<tr>
<td>9. 36/M</td>
<td>AR/BA</td>
<td>3+</td>
<td>5054</td>
<td>2.03</td>
<td>Rg, Sb</td>
<td></td>
</tr>
</tbody>
</table>

*AR, Allergy rhinitis; BA, Bronchial asthma; URT, Urticaria; GIS, Gastrointestinal symptoms.
M: Male; F: Female; IgE: Immunoglobulin E; SPT: Skin Prick Test.
Negative control for Specific IgE: 0.36.
Negative control for total IgE: 225-4305 IU/ml

**Abbreviated names of different legumes used in table:
Rg, Red gram; Sb, Soybean; Bn, Bean fresh; Bg, Bengal gram; Ln, Lentil; Ch, Chickpea
Fig. 5.1. (a) ELISA inhibition of green gram CPE.  
(b) ELISA inhibition of green gram with soybean ( — ), red gram ( — ), chickpea ( — ) and bengal gram ( — ) CPE.

Means (n=3) and SEs (bars) are represented.
treated group. The highest IgE levels were found on day 43 with slight decrease on day 59 (Fig. 5.2 a). Both green gram and peanut proteins elicited significantly high ($p<0.001$) specific IgE response on day 15, 43 and 59. Pattern of IgE response against green gram and peanut was similar and both the groups showed continuous increase in specific IgE levels up to 59 days (Fig. 5.2 b). Similar to peanut, green gram sensitized group showed significant increase in IgG1 levels ($p<0.001$) up to 59 days continuously (Fig. 5.3 a). In case of IgG2a, green gram showed more than 3 fold decrease on day 15, more than 6 fold decrease on day 43 and more than 5 fold decrease on 59 as compared to vehicle control, whereas peanut showed more than five fold decrease throughout (Fig. 5.3 b).

5.3.4. Antigen challenge of sensitized mice and histamine level
Hypersensitive symptoms became evident within 15 to 30 min after challenge. The most sever symptoms as expected were observed in positive control group of peanut sensitized mice with 80% mortality due to anaphylaxis. Mice sensitized with green gram also showed severe anaphylactic symptoms with 60% mortality (Fig. 5.4 a). However, control mice showed no symptoms of hypersensitivity after challenging with either peanut or green gram CPE. Histamine levels in plasma of peanut (9 fold) and green gram-sensitized mice (7 fold) were markedly increased after challenge with CPEs as compared to control animal (Fig. 5.4 b).

5.3.5. Histopathology of lungs, spleen and intestine
Green gram sensitized/challenged mice lung tissue revealed perivascular and peribronchial inflammatory cell infiltrate with narrowing of the bronchiolar lumen. Thickening of alveolar septa through-out the parenchyma were also observed. Mild bronchial epithelial hyperplasia was evident in both the green gram and peanut treated groups (Fig. 5.5 b and c).
Fig. 5.2. Serum immunoglobulin responses.
(a) Total IgE, and
(b) Specific IgE in sera of peanut and green gram-sensitized BALB/c mice.

Data are reported as means ± SEM for mice (n=5 per group). *** represent p value <0.001 as compared to control group.
Serum immunoglobulin responses.

(a) Specific IgG1, and
(b) Specific IgG2a in sera of peanut and green gram-sensitized BALB/c mice.

Data are reported as means ± SEM for mice (n=5 per group).
*** represent p values <0.001 as compared to control group.
The IgG2a levels have been presented in terms of fold decrease as compared to control.
Fig. 5.4. (a) Anaphylactic symptom scores in mice after peanut or green gram CPE challenge, and (b) Histamine levels in plasma.

Histamine is depicted means ± SEM for n=5 mice per group.

***P <0.001 values are significant as compared to control.
Fig. 5.5. Lung histology of sensitized/challenged mice. (a) PBS (control), (b) Peanut, (c) Green gram.

Perivascular and peribronchial inflammatory cell infiltrate is indicated with black arrow. Mild bronchial epithelial hyperplasia was evident in peanut treated groups (indicated with red arrow). Narrowing of the bronchiolar lumen in green gram treated group indicated with blue arrows.
However, lung from control group showed normal histological structure (Fig. 5.5 a).

Spleen from green gram sensitized/challenged group revealed lymphoid hyperplasia with activated macrophages like peanut treated group (Fig. 5.6 c). In addition, large megakaryocytes were observed in peanut treated group (Fig. 5.6 b). Spleen from control mice had normal histology of white pulp and red pulp (Fig. 5.6 a).

Unlike intestine of red gram treated mice, where goblet cell hyperplasia in submucosal layer and mucosal exfoliation were the important features, no histopathological changes were seen in green gram sensitized/challenged group (Fig. 5.7 c). Though positive control peanut sensitized/challenged group revealed intestinal pathological changes like infiltrations of inflammatory cells and mucosal exfoliation (Fig. 5.7 b). Intestine from control mice had normal histology (Fig. 5.7 a).

5.3.6. Cytokine responses to green gram CPE
A significant increase ($p<0.001$) in Th2 cytokines IL-4 and IL-10 was observed in splenocytes of green gram/peanut primed mice as compared to naïve mice (Fig. 5.8 a and b), whereas no significant difference was observed in the IL-2 level (Fig. 5.9 a). IFN-$\gamma$ levels, a Th1 cytokine was significantly decreased in green gram primed ($p<0.01$) splenocytes similar to peanut treated group (Fig. 5.9 b). These results demonstrate that green gram behaved in similar manner as peanut as it caused significant increase in IL-4, IL-10 and decrease in IFN-$\gamma$ production.

5.3.7. Changes in cytokine mRNA expression
Green gram CPE similar to peanut up regulated the Th2 cytokines like IL-4, IL-5 and IL-10 ($p<0.01$) at mRNA levels in whole blood and spleen of sensitized and challenged mice as compared to PBS treated mice (Fig. 5.10 a
Fig. 5.6. Spleen histology of sensitized/challenged mice. 
(a) PBS (control), (b) Peanut, (c) Green gram.

Lymphoid hyperplasia (indicated by black arrow) and large sized megakaryocytes are indicated by red arrows.
Fig 5.7. Intestine histology of sensitized/challenged mice. (a) PBS (control), (b) Peanut, (c) Green gram.

Heavy infiltrations of inflammatory cells and mucosal exfoliation are indicated by black arrows.
Fig. 5.8. Effect of green gram CPE on Th2 cytokine levels in splenocyte culture supernatants.
(a) IL-4, and
(b) IL-10 levels were measured by ELISA.

Data are presented as means ± SEM.
*** $p<0.001$ values are significant as compared to control.
Fig. 5.9. Effect of green gram CPE on Th1 cytokine levels in splenocyte culture supernatants. (a) IL-2, and (b) IFN-γ levels were measured by ELISA.

Data are presented as means ± SEM. ** p<0.01 and *** p<0.001 values are significant as compared to control.
(a) Blood

(b) Spleen

Fig. 5.10. Effect of green gram treatment on Th2 cytokine mRNA expression in whole blood and spleen. Th2 cytokines included IL-4, IL-5 and IL-10 in (a) blood and (b) spleen. β-actin was taken as endogenous control. Data are means ± SEM for 5 mice in each group. Graph was plotted taking the value of relative intensity of cytokine versus β-actin.
and b). Green gram and peanut CPE treated groups did not show any significant change in IL-2 mRNA levels as compared to PBS treated mice both in blood as well as spleen. In whole blood, reduction in IFN-γ level was significant \( (p < 0.01) \) in peanut treated group only. However, significant down regulation of Th1 type cytokine IL-12 \( (p < 0.001) \) and IFN-γ \( (p < 0.01) \) at mRNA level was observed in spleen of both green gram and peanut treated groups (Fig. 5.11 a and b).

5.3.8. SGF digestibility and allergenic proteins of green gram CPE
SDS-PAGE of green gram CPE resulted in proteins ranging from 18 to 170 kDa (Fig. 5.12 a, lane 2). Five proteins of 52, 38, 35, 30 and 18 kDa were stable up to 2 min (Fig. 5.12 a, lane 4) following SGF digestion. IgE immunoblot of green gram CPE with pooled patient’s sera revealed five IgE binding proteins of 52, 38, 35, 30 and 18 kDa (Fig. 5.12, b). The IgE reactivity of green gram CPE was also analysed by individual patient’s serum. The 52 kDa protein was the most frequent IgE-binding protein which was detected by immunoblots with all the patient’s sera, while proteins of 38 and 35 kDa recognized by 44%, 30 kDa by 67% patients’ sera and 18kDa were recognized by 22% of patients’ sera, respectively. The strips incubated with NHS did not show any band. Green gram extract was also incubated with PBS and showed no nonspecific binding. These results clearly demonstrate that pepsin sensitive proteins did not show any IgE binding, whereas pepsin stable proteins showed IgE binding.

5.3.9. Identification and characterization of IgE binding proteins of green gram
IgE specific immunoblotting of 2DE resolved green gram CPE with pooled sera of nine patients (having SPT positive and high levels of specific IgE to green gram protein extract) showed 6 IgE binding protein spots of 52, 50, 38,
(a) Blood

![Graph showing relative intensity of cytokines in blood samples.]

(b) Spleen

![Graph showing relative intensity of cytokines in spleen samples.]

**Fig. 5.11.** Effect of green gram treatment on Th1 cytokines mRNA expression in whole blood and spleen. Th1 cytokines included IL-2, IL-12 and IFN-γ in (a) blood and (b) spleen. β-actin was taken as endogenous control. Data are means ± SEM for 5 mice in each group. Graph was plotted taking the value of relative intensity of cytokine versus β-actin.
Fig. 5.12. SGF digestion and immunoblot of green gram CPE.

(a) Lane 1: Pepsin, Lane 2: green gram CPE; Lane 3: SGF digestion pattern of green gram at 0 minutes; Lane 4: SGF digestion pattern of green gram at 2 minutes; Lane 5: Molecular weight markers.

(b) Lane PS: Immunoblot of green gram CPE with pooled sera (1:20) of green gram sensitive patient; Lane 1-9: Immunoblot of green gram CPE with individual serum of green gram CPE sensitive patients. Lane NHS: Immunoblot of green gram CPE with pooled serum from 10 patients, allergic to dander and insects but not to any food or pollen extracts was used as negative control; Lane PBS: Green gram CPE incubated with PBS and HRP linked anti-human IgE (1:1000).
35, 30 and 18 kDa (Fig. 5.13, c). Fig. 5.13 b, shows that sera from green gram sensitized mice recognized the four IgE binding proteins of 52 kDa (~pI 5.65), 50 kDa (~pI 5.79), 30 kDa (~pI 6.58) and 18 kDa (~pI 5.53), named as Gg1, Gg2, Gg3 and Gg4, respectively for the sake of convenience. These four proteins were further subjected to mass spectrometry analysis. Taken together, these results indicate that the 52, 50, 30 and 18 kDa green gram proteins may be responsible for inducing allergenic reaction in humans and perhaps induce such reactions in BALB/c mice as well.

5.3.10. Mass spectrometric analysis of the four IgE binding proteins

On the basis of significantly high MOWSE score, mass spectrometric analysis revealed Gg1, Gg2 and Gg3 protein spots as 8S globulin β isoform precursor, 8S globulin α isoform precursor and seed albumin of green gram, respectively (Table 5.2). Individual ion scores indicated identity or extensive homology and protein scores were derived from ion scores as a non-probabilistic basis for ranking protein hits. Protein scores greater than 45 were considered as significant. The probability based MOWSE score obtained for 8S globulin β isoform precursor (Gg1), 8S globulin α isoform precursor (Gg2) and seed albumin (Gg3) was 94 (p<0.05) with maximum 10 hits and 8 queries match, 236 (p<0.05) with maximum 15 hits and 9 queries match and 45 (p<0.05) with maximum 19 hits and 8 queries, respectively. Tryptic digestion of Gg4 spot followed by LC-MS/MS analysis of the resultant peptides showed homology with canavalin of sword bean (Table 5.2, Fig 5.14 a-d).

The tryptic fragment KQIQNLENYRVVEFKS of Gg1 protein corresponded to amino acid 75-90, RAILTLVNPDR to 113-125, KLAIPVNNPHRF to 158-169, KELSSQDEPFNL to 248-260, and RWYEITPEKLPQKLIDVFISSVDMKEGLLLLPHYS to 271-309 of 8S
Fig. 5.13. (a) Two-dimensional (2D) SDS-PAGE profile of green gram CPE; (b) IgE binding proteins detected after immunoblotting of 2D resolved green gram CPE with pooled serum of green gram sensitized mice; (c) IgE binding proteins detected after immunoblotting of 2D resolved green gram CPE with pooled serum of patients allergic to green gram. *The spots analysed by trypsin-digestion and mass spectrometry are outlined by solid ellipses and named as Gg1, Gg2, Gg3 and Gg4.
Table 5.2: Mass spectrometric analysis of IgE binding proteins of green gram

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Protein Designation</th>
<th>Tryptic Peptide Fragments</th>
<th>Sequence Similarity</th>
<th>Score</th>
<th>Expected Mol wt (kDa)</th>
<th>Calculated Mol wt (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Gg1</td>
<td>KLAIVNNPHRF</td>
<td>8S globulin beta isoform precursor-Vigna radiata</td>
<td>94</td>
<td>52</td>
<td>51.75</td>
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<td></td>
<td></td>
<td>RAILTLVNPDPGRD</td>
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<td>2.</td>
<td>Gg2</td>
<td>RNQFGHRLRVEQIRELTKH</td>
<td>8S globulin alpha isoform precursor-Vigna radiata</td>
<td>236</td>
<td>50</td>
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<tr>
<td></td>
<td></td>
<td>KNILEASFSDSKEISRVE</td>
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<tr>
<td></td>
<td></td>
<td>RDLDFRISVDMKEGSL</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>KKSLSSEDQPFNLNRQK</td>
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<td>PIYSNKL</td>
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<tr>
<td></td>
<td></td>
<td>RNFLAGEKDNWISEEIPTEVL</td>
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<td></td>
<td>DVTTPASGEKV</td>
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<tr>
<td>3.</td>
<td>Gg3</td>
<td>RLQYTPGKTTILAGPITTAEMFVPLRN</td>
<td>Seed albumin-Vigna radiata</td>
<td>148</td>
<td>30</td>
<td>30.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNTVFAIDSAFPRSKGEVYLFKG</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>RIDYDSKQVGSIRNRIHFTPNGKT</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>4.</td>
<td>Gg4</td>
<td>KTLSSQDKPFLNRSE</td>
<td>Canavalin-Sword bean</td>
<td>60</td>
<td>18</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 5.14. Bar diagrams showing LC/MS-MS results of different green gram IgE binding proteins. LC/MS-MS mass spectra and probability plot of (a) Gg1, (b) Gg2, (c) Gg3 and (d) Gg4 protein spots of green gram. High probability score were used to identify proteins. Mass tolerance (Peptide mass tolerance of ±2 Da) and monoisotopic values were used for searching.
globulin β isoform precursor (Fig. 5.15). Peptide fragment RNQFGHLRV of Gg2 corresponded to amino acid 60-68, RSKMQNLEN YRV to 75-87, KNILEASF DSIKEISRV to 191-208, REQUIRELTKH to 234-243, KKSLSEDQPFNLRNQKPIY SNKL to 249-272, RDLDSEIRSVDMK EGSLLLLPHYNSKA to 286-312 and RNFLAGKDNVISEI PTEVLDTVP ASGEKV to 389-419 position of 8S globulin α isoform precursor (Fig. 5.16), whereas Gg3 tryptic digested peptide fragment RLQYTPGKT corresponded to amino acid 30-38, KILAGPTTIAEMFPVRNT VFADSIDSAFRS to 98-128, KGKEVYLFKG to 130-139, RIDYDSKQLV GSIRN to 144-158 and RIHFTPGKT to 197-205 position of seed albumin of green gram when alignment was carried out (Fig. 5.17).

5.3.11. Sequence analysis
According to the positions of tryptic digested peptide sequences of respective green gram proteins on the amino acid sequence of protein with maximum ion score, the order of tryptic fragments were arranged. Ordered tryptic fragment sequences of all these protein spots were arranged with CLUSTAL W and subjected to BLAST search against NCBInr protein database to confirm their identity. 8S globulin β isoform precursor, 8S globulin α isoform precursor and seed albumin proteins are not identified as an allergen prior to this study, as these proteins are not present in different databases (like allergen Online, structural database of allergenic proteins, Food Allergy Research and Resource program) of allergenic proteins. To assess whether these IgE binding proteins were having any similarity to other allergens, their ordered amino acid sequences were subjected to over all FASTA search on allergen-online database. Upon full length FASTA search where identity matches greater than 50% indicating possible cross-reactivity, 8S globulin β isoform precursor (Gg1) and 8S globulin α isoform
Fig. 5.15. Alignment of the Ggl green gram protein sequences obtained from LC-MS/MS analysis with full length sequence of 8S globulin β-isoform precursor of green gram

Ggl
BWB23689

MVRARVQLLLLGLILFLASLSVSFGIVHREHGESQESDSRGQNNPYFNSDRRFHTLFKNQ

Ggl
BWB23689

KQIQNLENYRVVEFKSKQIQNLENYRVVEFKSKPNTLLLPHHADADFLLVLNGRAILTLVN

Ggl
BWB23689

YGLRLVHRFQRSKKQIQNLENYRVVEFKSKPNTLLLPHHADADFLLVLNGRAILTLVN

Ggl
BWB23689

PDGRD---------KLAIPVNNPHRFK---------

BWB23689

PDGRDYSYIEQHAQKIPAGTFFFLVNPNDNDLNRIIKLAIPVNNPHRFQNFFLSSTEAQ

Ggl
BWB23689

QSYLRGFSKNILEAFSDFKTEDRVLFGEERQQHGEESEQEVIVELKREQIREL

Ggl
BWB23689

-------------------KELSSQDEPFNLRN---------------------RWEITPEKNPQKDLDFVISSVDMKEGGL

BWB23689

AKSSSRKELSSQDEPFNRLNSNPIYSNKFGRWYEITPEKNPQKDLDFVISSVDMKEGGL

Ggl
BWB23689

LLPHYNSKA-----------------------------

BWB23689

LLPHYNSKAINILVINEGAKIELVGPSPQQQDESLVQRAYRAELSEDVVFVIPAAPYV

Ggl
BWB23689

-----------------------------

BWB23689

AINATSNLNFFAFGIANQNRNFAGKDNMSEIPETVLDSFPASGNKVEKLKQ

Ggl
BWB23689

-----------------------------

BWB23689

ESHFVDAQPEQQQREEGHKGRKGSLLSGSLY

**Ordered fragment:**

KQIQNLENYRVVEFKSRAILTLVNPDRDKLAIPVNNPHRFKELSSQDEPFNLRNRWYEITPEKNPQKDLDFVISSVDMKERGLLPHYNKSA
Fig. 5.16. Alignment of the Gg2 green gram protein sequences obtained from LC-MS/MS analysis with full length sequence of 8S globulin α isoform precursor of green gram

Ordered Fragment:

RNQFGHLRVRSKQMQLENYRVKNILEASFSDIKIEISRVRQIRELTKHKSSLSEDQPFNLRNQKPIYSNKLRLDLMFIRSVDMKEGSLLLPHYNSKARNFLAGEKDNVISEIPTEVLDTVPASGEKV
Fig. 5.17. Alignment of the Gg3 green gram protein sequences obtained from LC-MS/MS analysis with full length sequence of seed albumin of green gram

Gg_3
BWB9894

MSNLPYINAARFSSRDYEVYFFAKNYVRKQYTPKTEDKILTNLISGFSPLAGTP

Gg_3
BWB9894

FAEPGIDSAGHTAEASEAYVFSANNRAIDYPGTNDKILAGPTTIAEMFPVLRNTVFAD

Gg_3
BWB9894

SIDSAFRSKGKEVFGLKMKYVRIDYDQVLGSRINISDFPVNLNTGFESGIDASFA

Gg_3
BWB9894

SHKEPEAYLFKGDKHYVRIHFPGKTDDTLVGDVRPILDGWVILKAFCLELNKPSLSCIN

Gg_3
BWB9894

HLSTVITINKAFISNVCLFFNVTLGLEACFLS

Ordered Fragment:

RLQYTPGKTIKRAMPTTIAEMFPVLRNTVFADSIDSAFRSKGKEVFGLKMKYVRIDYDQVLGSRINRHIHFPGKT
precursor (Gg2) showed homology with other known allergenic proteins like β-conglycinin-α subunit and β-conglycinin storage protein of soybean, allergen Len c 1.0101 and Len c 1.0102 of lentil, vicilin of pea, Ara h1 of peanut and conglutinin β of lupin (Table 5.3). Ordered fragments of seed albumin (Gg3) showed sequence homology to seed storage protein 13 S globulin of buck wheat and Bet v 5 of birch pollen allergen.

BLAST search of 8S globulin β isoform precursor and 8S globulin α isoform precursor amino acid sequence on NCBI database for conserved domain showed that both of these proteins are member of cupin superfamily and contain two cupin conserved domains (Fig. 5.18).

5.4. Discussion
Green gram is consumed worldwide, mainly in south-east Asia due to its high protein content. However, knowledge about allergic responses as well as its clinically relevant allergens is lacking. In chapter 3, we have reported that green gram is one of the potent sensitizing agents by virtue of being the major source of food protein. Therefore, the present study was undertaken to evaluate the allergenic potential of green gram using animal model and the allergens present therein were identified and characterized. In light of history, SPT and specific IgE ELISA against green gram, sensitization to this legume was observed in 9 naso-bronchial allergic patients. Significantly elevated green gram specific IgE levels evince that these patients are sensitized to green gram. An earlier study of SPT positive chickpea patients showed raised IgE levels corroborating our findings (Patil et. al, 2001).

To elucidate the mechanism of green gram induced allergy, BALB/c mice were sensitized with green gram CPE. Food allergy for the most part, is mediated by allergen specific IgE antibodies that induce Th2 response. IgG1 antibodies are also associated with Th2 cells, whereas IgG2a induction
Table 5.3: Sequence homology analysis of tryptic fragments of green gram IgE binding proteins

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Protein</th>
<th>Tryptic Peptide Fragments</th>
<th>Ordered tryptic fragments</th>
<th>Sequence similarity using Full FASTA search</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Gg1</td>
<td>KLAIPVNNPHRF RAIIIVNFPGDR</td>
<td>KQIQNLERNYRVE KSKAIITLVNPDP</td>
<td>β-conglycinin-α subunit (Glycine max)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KEQGKLPHYN SKA</td>
<td>RKSLKQDEEFNLPR LKDVLFPSSVD</td>
<td>β-conglycinin storage protein (Glycine max)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KELSSQDEPFNKL RNWYETTPKNPQ</td>
<td>LKSLDLLVPSSVDM KERGGLPHYN SKA</td>
<td>Allergen Len c 1.0101 (Lens culinaris)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KEQGKLPHYN SKA</td>
<td>RKSLKQDEEFNLPR LKDVLFPSSVD</td>
<td>Allergen Len c 1.0102 (Lens culinaris)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KEQGKLPHYN SKA</td>
<td>RKSLKQDEEFNLPR LKDVLFPSSVD</td>
<td>Vicilin (Pisum sativum)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KEQGKLPHYN SKA</td>
<td>RKSLKQDEEFNLPR LKDVLFPSSVD</td>
<td>Conglutin β (Lupinus angustifolius)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KEQGKLPHYN SKA</td>
<td>RKSLKQDEEFNLPR LKDVLFPSSVD</td>
<td>Ara h1 (Arachis hypogaea)</td>
</tr>
<tr>
<td>2.</td>
<td>Gg2</td>
<td>RNNQFGHLRV RQIRELTKH</td>
<td>RNQFGHLRVRS KMQNLENYRVE</td>
<td>β-conglycinin-α subunit (Glycine max)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSKMQQNLENY RV</td>
<td>RNQFGHLRVRS KMQNLENYRVE</td>
<td>β-conglycinin storage protein (Glycine max)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KNILEASFSDIK EISRV</td>
<td>RNQFGHLRVRS KMQNLENYRVE</td>
<td>Allergen Len c 1.0101 (Lens culinaris)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RSDLDFSRVD NSKA</td>
<td>RNQFGHLRVRS KMQNLENYRVE</td>
<td>Allergen Len c 1.0102 (Lens culinaris)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KKSLSSEDQFPFLN RNKPIYSNK</td>
<td>RNQFGHLRVRS KMQNLENYRVE</td>
<td>Vicilin (Pisum sativum)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RNFLAGEKDNV ISEITPEVDVT</td>
<td>RNQFGHLRVRS KMQNLENYRVE</td>
<td>Conglutin β (Lupinus angustifolius)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PASEGKV</td>
<td>RNQFGHLRVRS KMQNLENYRVE</td>
<td>Ara h1 (Arachis hypogaea)</td>
</tr>
<tr>
<td>3.</td>
<td>Gg3</td>
<td>RLOQTPGKTE</td>
<td>RLOQTPGKTKILA GPTIAEMFVPLR</td>
<td>Seed storage protein / (Buck wheat; Fagopyrum esculentum)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KILAGPTIAEM FVLPNR</td>
<td>RLOQTPGKTKILA GPTIAEMFVPLR</td>
<td>Bet v 5 (Birch pollen allergen)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KNIFVADSIDS AFR</td>
<td>RLOQTPGKTKILA GPTIAEMFVPLR</td>
<td>13 S globulin / (Buck wheat; Fagopyrum esculentum)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KGKEVLFKGR</td>
<td>RLOQTPGKTKILA GPTIAEMFVPLR</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>RIDYDSKQLVGS IRN</td>
<td>RLOQTPGKTKILA GPTIAEMFVPLR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RIHFPGKT</td>
<td>RLOQTPGKTKILA GPTIAEMFVPLR</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>Gg4</td>
<td>KTLSSQKPFNKLRS</td>
<td>KTLSSQKPFNKLRS</td>
<td>β-conglycinin-α subunit (Glycine max)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td>β-conglycinin storage protein (Glycine max)</td>
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<td>Allergen Len c 1.0101 (Lens culinaris)</td>
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<td>Allergen Len c 1.0102 (Lens culinaris)</td>
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<td></td>
<td>Vicilin (Pisum sativum)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Conglutin β (Lupinus angustifolius)</td>
</tr>
</tbody>
</table>
Fig. 5.18. Analysis of conserved domain of Gg2 using BLAST search. Gg2 shares conserved domain with Cupin superfamily.
requires Th1 cells (Abbas et al., 1996). Stasis between IL-4 and IFN-γ decide the levels of these immunoglobulin. IFN-γ promotes IgG2a production and inhibits IgE responses (Coffman and Carty, 1986; Snapper et al., 1988), whereas IL-4 enhances IgE and IgG1 production and inhibits IgG2a production. Green gram administration results in up regulation of Th2 cytokines including IL-4. Levels of Th1 cytokines both at mRNA as well as protein levels were significantly reduced except IL-2 where no change was observed in green gram treated animals. This modification in levels of Th2 and Th1 cytokines are in coherence to increased IgE and IgG1 levels and decreased IgG2a levels in sera of green gram sensitized mice. Green gram up regulated Th2 cytokines and down regulated Th1 cytokines like IFN-γ and IL-12 significantly, though IL-2 levels remained unaffected. In our study, green gram induced high IgE response similar to peanut. Taken together, these data revealed that green gram is having allergenic potential comparable to peanut.

Histamine plays an important role in the pathogenesis of allergy process (Galli, 1993) and augments the release of Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13 and lowers Th1 cytokines IL-2, IFN-γ and IL-12 release (Elenkov et al., 1998; Sirois et al., 2000). The development of allergic reactions is associated with excessive histamine production (Elenkov et al., 1998). Upon allergen mediated cross linking of mast cells bound IgE; histamine and other mediators are released and contribute to the symptoms of anaphylaxis. This explains severe anaphylactic symptoms and manifold increase in plasma histamine level upon challenge with green gram sensitized mice.

Airway hyper-responsiveness is unremarkably observed in asthma an immune-inflammatory disease that is characterized by peribronchial recruitment of inflammatory cells (O'Donnell et al., 1998). It was observed that green gram mice on challenge exhibited signs of anaphylaxis leading to
death. Lung sections of green gram sensitized/challenged mice revealed build up of inflammatory cells in the perivascular and peribronchial regions of the lung parenchyma with contraction of bronchiolar vacuoles. Lungs histology further confirmed that exposure to green gram increased eosinophils and caused airway blockage. The lymphoid hyperplasia noticed as the typical histological attribute of the spleens relevant to allergic response perhaps indicate massive proliferation and differentiation of T lymphocytes which are main histological signs of acute or chronic inflammation in an organ (Ryan et al., 1977). These, histological changes supported the modulation in cytokines and immunoglobulin levels; undoubtedly pinpointing allergenic potential of green gram.

The significant factor responsible for allergenicity of food proteins is their stability against pepsin digestion (Astwood et al., 1996; Besler et al., 2001; Thomas et al., 2004). In chapter 3, data clearly demonstrate that proteins stable in SGF for >2 min in case of soybean and chickpea showed ability to bind with specific IgE of allergic patient’s sera. On in vitro pepsin digestion of green gram CPE, 52, 38, 35, 30 and 18 kDa proteins were found to be resistant to SGF digestion after 2 min. It was inferred therefore, that these SGF stable proteins have potential for absorption by the intestinal mucosa and hence the chances of these to behave as an allergens are more. It was clearly shown that all these proteins had specific IgE binding capacity when immunoblotted with sensitized patient’s sera, confirming allergenicity of these pepsin resistant proteins. 2DE resolved electroblot showed six IgE binding proteins of 52, 50, 38, 35, 30 and 18 kDa when incubated with patient’s sera. However, sera from sensitized mice recognized only four proteins of mol wts 52, 50, 30 and 18 kDa. The proteins recognized by both human and mouse sera were named as Gg1 (52 kDa), Gg2 (50 kDa), Gg3 (30 kDa) and Gg4 (18
kDa), respectively, and were further characterized by mass spectrometry (where Gg stands for Green gram).

Mass spectrometric analysis indicated that Gg1 protein is probably, 8S globulin β isoform precursor, Gg2 is 8S globulin α isoform precursor and Gg3 is seed albumin from green gram with high probability based MOWSE score. Gg4 did not show any significant similarity with any known green gram protein, though it showed significant match to conavalin of sword bean. β and α isoform precursors of 8S globulin are major seed storage protein of green gram and belong to cupin superfamily, a ubiquitous protein, as cupin conserved domain is present in amino acid sequence of these two proteins. Cupin superfamily consists of seed storage proteins and a number of cupins have been identified as major plant food allergens, including the 7 S globulins of soybean (β-conglycinin), peanut (conarachin; Ara h 1), lentil (Len c1), 11 S globulins of peanut (arachin; Ara h 3) etc (Mills et al., 2002). FATSA search of Gg1, Gg2 and Gg3 proteins for sequence matching showed significant homology with known allergenic cupin proteins. These results indicate that there is sufficient structural and significant sequential homology between green gram and other legume seed storage protein allergens. ELISA inhibition results showed strong cross-reactivity between soybean corroborating LC/MS-MS findings, indicating the probable IgE binding epitope sharing and phylogenetic relation with soybean proteins (Ibáñez et al., 2003; Ireneo et al., 2008).

The study supports the allergenic potential of green gram proteins as its administration induces Th2 responses and decreases Th1 responses in mice. Moreover, immunoblot with green gram allergic patient’s sera as well as sensitized mice sera revealed four IgE binding proteins having mol wts of 52, 50, 30 and 18 kDa, as putative clinically relevant allergens (Fig. 5.19) Identification of allergens from a pulse like green gram will go a long way in
Fig. 5.19. Summary of green gram protein induced allergenic responses
Chapter 5

taking prudence by hypersensitive patients showing allergy to legumes. The recognition of new food allergens is the initial step towards future diagnostic and therapeutic operandi to deal cogently with food allergy. The identified allergens could be mutated to obtain hypoallergenic molecules of potential use for immunotherapy and also generate transgenic green gram that may be better and less allergic than the present native crop available in the market.

5.5. Conclusion

These results suggest that green gram has allergenic potential and contain allergenic proteins that are capable of inducing IgE-mediated reactions in respective legumes sensitized patients as well as in BALB/c mice. We identified four novel allergens in green gram and christened them Gg1, Gg2, Gg3 and Gg4. LC-MS/MS revealed that Gg1, Gg2 and Gg3 proteins are 8S globulin β, α isoform precursors and seed albumin, respectively. Detailed characterization is required to establish these green gram proteins as allergens.