

ORIGINAL ARTICLE

Potential allergens of green gram (*Vigna radiata* L. Millsp) identified as members of cupin superfamily and seed albumin

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Summary

Background No systematic study on allergenicity of green gram seed proteins have been performed so far, although incidences of IgE-mediated reaction to green gram seedlings have been reported.

Objective We sought to investigate the allergenic potential of green gram, followed by identification and characterization of its relevant allergens using proteomic approaches.

Methods BALB/c mice were sensitized intraperitoneally with green gram proteins, and levels of specific Igs, Th2 cytokines, histamine, anaphylactic symptoms and histopathological responses were studied. Twelve naso-bronchial allergic patients with a history of sensitization to green gram were selected on the basis of positive skin prick test and elevated specific IgE levels. Green gram allergens were identified and characterized by their ability to endure pepsin, by IgE immunoblot of two-dimensional (2D) gels in combination with mass spectrometry and by bioinformatics approaches.

Results Increased specific IgE, IgG1, Th2 cytokine and histamine levels, high anaphylactic scores and histological changes in lungs and spleen of green gram crude protein extract-treated mice are indicative of its sensitization ability. Four proteins (molecular weights: 52, 50, 30 and 18 kDa) showed pepsin resistance and IgE-binding capability with sensitized human and mice sera. The four proteins tentatively named as Vig r2 (52 kDa, pI 5.7), Vig r3 (50 kDa, pI 5.8), Vig r4 (30 kDa, pI 6.6) and Vig r5 (18 kDa, pI 5.5) showed significant sequence similarity with known allergens of soybean, lentil, pea, lupin, etc. Mass spectrometric analysis identified Vig r2 as 8S globulin β -isoform precursor, Vig r3 as 8S globulin α -isoform precursor and Vig r4 as seed albumin.

Conclusion and Clinical Relevance Green gram seeds contain at least four clinically relevant allergenic proteins, namely Vig r2, Vig r3, Vig r4 and Vig r5 that were capable of inducing strong IgE-mediated reactions. One of the most important steps towards diagnostic and therapeutic approaches to deal effectively with food allergy is continued identification of newer food allergens and their characterization. The significance of this study can be enormous as the data generated may work as basic biology data in developing a green gram species modified genetically that may have reduced allergenicity.

Keywords 8S globulin, food allergy, green gram, IgE-binding proteins, leguminous crops, novel allergens, seed storage proteins

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Introduction

Green gram (*Vigna radiata* L. Millsp, family Leguminosae), a native of India, is commonly known as 'mung bean'.

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It is widely cultivated throughout Asia, including Pakistan, Bangladesh, Sri Lanka, Thailand, Laos, Cambodia, Vietnam, Indonesia, Malaysia, China and Formosa. Mung bean is a protein-rich staple food, containing about 24% protein, which is almost three times that of cereals [1]. This legume is consumed in the form of whole as well as split pulse and supplements cereal-based diet. Green gram

is also an excellent source of vitamins, minerals and proteins comparable with that of soybean and kidney bean [2]. Antifungal and antibacterial activity of certain proteins of green gram is well documented [3].

The utilization of green gram for human nutrition may be constrained by the presence of inherent anti-nutritional factors such as phytic acid, polyphenols and protease inhibitors, which are known to reduce the availability of nutrients [4, 5]. The presence of protease inhibitors is known to abate the digestibility of legume proteins [6–9]. Resistance to digestion against gastric enzymes is thought to be an important attribute of allergenic food proteins [10–13]. Amalgam of gastric enzyme-resistant proteins and the presence of protease inhibitors may increase chances of proteins (s) entering the intestine undigested, thereby enhancing intestinal immune system exposure, and results in allergenic response. Earlier, we have reported green gram sensitization in naso-bronchial allergic patients [14]. These patients reported nausea, abdominal pain, irregular bowels, itching, and urticaria after consumption of green gram. The observed clinical symptoms are reminiscent of an allergic manifestation in susceptible patients on green gram consumption. Moreover, a protein from green gram seedling, Vig r1 had been identified as an allergen by Mittag et al., [15]; however, no allergens from green gram seed, the major eatable part, are yet reported. Its accompaniment with allergy to soybean and birch pollen seems to be common implying cross-reactivity [14, 15].

Therefore, the present study was undertaken to assess the allergenic potential of green gram in BALB/c mice and then further explicated to naso-bronchial patients sensitive to green gram. Identification of allergens may be helpful in the development of non-allergenic transgenic variety and characterization may help in the immunotherapy and clinical screening. Therefore, the principal objective of the current investigation was to identify and characterize the allergenic proteins using proteomics and bioinformatics tools.

Methods

Test material and reagents

All the chemicals were of highest grade purity available. Green gram seeds were purchased from a local certified seed vendor and the same lot was used throughout the study. The K851 variety released in Central India was chosen for study. The variety with notification number 19(E) was notified on January 14, 1982. The dry seeds were stored at 5–8° C and 25–30% relative humidity. Seeds were put in water-proof container with coloured silica gel desiccant as an indicator of dryness.

Preparations of green gram crude protein extract. Crude green gram protein extract was prepared as described by

Astwood et al. [10] with slight modifications as mentioned earlier [16].

In vitro digestibility of green gram crude protein extract. *In vitro* simulated gastric fluid (SGF) digestibility was performed as described by Astwood et al. [10] with slight modifications as mentioned earlier [16]. Green gram crude protein extract (CPE) was digested and subjected to reproducible SDS-PAGE.

Preparation of simulated gastric fluid. Pepsin (3.2 mg) (approximately 3460 U activity/mg) was dissolved in 1 mL of 34 mM NaCl and 0.7% HCl, pH 1.2. SGF solution was freshly prepared and used within the same day.

Digestibility of green gram crude protein extract. SGF assay was performed taking 4 mg/mL green gram CPE. SGF (430 µL) was incubated at 37 °C before the addition of 86 µL of test protein solution. The contents of the tube were mixed by mild vortexing and the tube was immediately placed in a 37 °C water bath. The reaction was stopped at different time intervals (0.25, 0.5, 1, 2, 4, 8, 15 and 60 min) by the addition of 60 µL of the incubation mixture to 60 µL of stopping solution, that is 2× Tris-tricine SDS sample buffer (containing 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol and 100 mM Tris, pH 8.8). SGF and green gram CPE were added directly to the stopping solution before the incubation for 0 min control. Each sample was boiled for 5 min at 100 °C. Boiled samples (15 µL/well) were subjected to reproducible SDS-PAGE (14%). Coomassie brilliant blue (R-250) stain was used for the gels. Gel images were captured using Syngene Bio Imaging System (Syngene, Cambridge, UK).

Animal studies

Healthy 6–8-week-old female BALB/c mice (22±3 g) were obtained from the IITR, Lucknow, India animal breeding colony. The mice were housed in the animal care facility of IITR, Lucknow, under standard laboratory conditions. The Animal Ethics Committee of the IITR approved the study protocol.

Animal study protocol. Mice were randomly divided into three groups ($n = 15$ /group). Group 1 received 100 µL of phosphate-buffered saline (PBS) (negative control), group 2 was administered peanut (positive control) and group 3 received green gram CPE (test group). Mice ($n = 15$) were injected intraperitoneally (i.p.) with 100 µg of protein in 100 µL of PBS once a week. Blood samples were collected from retro-orbital sinus to measure serum IgE and IgG1 antibodies on day 15, 43 and 59. On day 15, a subgroup of mice ($n = 5$ /group) were killed for splenocyte culture. Another subgroup of mice ($n = 5$ /group) were killed on day 15 and their blood and spleen were collected for RT-

PCR analysis after challenge with 10 mg of CPE for cytokine mRNA. On day 60, the remaining mice ($n = 5$ /group) were challenged i.p. with 1 mL of 10 mg/mL CPE. Tissue and blood samples were collected for histopathology and histamine assay, respectively. Specific IgE, IgG1 and cytokine levels were measured twice in triplicate. Histamine levels were measured once in triplicate (supporting information Fig. S3).

Assessment of systemic anaphylactic signs. Anaphylactic signs were evaluated 30–40 min after challenge by two investigators using the scoring system described previously by Li et al. [17], where 0 = no signs; 1 = scratching and rubbing around the snout and head; 2 = puffiness around the eyes and snout, diarrhoea, pilar erecti, reduced activity and/or decreased activity with increased respiratory rate; 3 = wheezing, laboured respiration and cyanosis around the mouth and the tail; 4 = no activity after prodding, or tremor and convulsions; and 5 = death.

Measurement of plasma histamine levels

Plasma histamine levels were determined 30 min after challenge by means of an ELISA kit (SPI-BIO, Montigny le Bretonneux, France) following the manufacturers' instructions.

Protein-specific immunoglobulin E and G1 estimation in mice. Serum antibodies specific for green gram and peanut CPE were measured by indirect ELISA [18]. Briefly, the well of microtiter plate (Nunc, Roskilde, Denmark) was coated with 1 µg of protein. After blocking, the plates were incubated with mice sera for IgE estimation (1 : 10) and IgG1 (1 : 1000) for 2 h at 37 °C. After washing, horseradish peroxidase (HRP) conjugated anti-mouse IgE and IgG1 antibodies (1 : 1000; Southern Biotech, Birmingham, AL, USA) were added and incubated for 1 h at 37 °C. Colour was developed with OPD and optical density was determined at 492 nm.

Cytokine reverse transcriptase-polymerase chain reaction. A semi-quantitative RT-PCR analysis of Th2 cytokines (IL-4, IL-5 and IL-10) in blood and spleen of mice was performed taking gene-specific primers. Sense and anti-sense primer sequences of IL-4, IL-5 and IL-10 were similar to Misra et al. [19]. Total RNA from blood and spleen was isolated with Tri BD and Tri Reagent (Sigma Chemical Co., St. Louis, MO, USA), respectively, according to the manufacturers' instructions. Total RNA was treated with RNase-free DNase (Fermentas, Glen Burnie, MD, USA) and its integrity was determined by 1.2% agarose gel electrophoresis before further downstream application. The RT-PCR was carried out using a One-Step RT-PCR kit (QIAGEN, Venlo, the Netherlands) and 1 µg of total RNA as template for each reaction. β -actin gene was

selected as endogenous internal standard. The thermal cycler (G-Storm, Essex, UK) was programmed as follows: reverse transcription at 50 °C for 30 min and subsequent 40 cycles of 94 °C denaturation for 30 s, 55 °C annealing for 30 s and extension at 72 °C for 30 s, with final extension at 72 °C for 10 min. After completion of PCR cycles, 10 µL of PCR product was analysed on 2% agarose gel electrophoresis. The density of each band was estimated by the Genetools software (Syngene). β -actin was taken as an endogenous control and its respective densitometric values were used for normalizing different mRNA cytokines in blood and spleen separately. Normalized values were used for plotting the bar graphs.

Spleen cell culture and cytokine measurements in supernatant of splenocyte culture. Spleen cell suspensions were prepared aseptically in RPMI-1640 (Sigma Chemical Co.), according to O'Donnell and Openshaw [20] with slight modifications. Splenocytes were seeded (1×10^6 cells/well) in triplicate with 100 µg/mL green gram or peanut CPE (primed) or without CPE (taken as control to primed splenocytes). The supernatants from cultured splenocytes were collected after centrifugation at 100 *g* for 5 min, at 24, 48 and 72 h and stored at –80 °C until analysis. Mouse IL-4 and IL-10 levels in culture supernatants were determined using an ELISA kit (eBioscience, San Diego, CA, USA).

Histopathological studies

After complete necropsy, lungs and spleen (4 mice/group) were washed in cold normal saline solution, fixed in 10% buffered formalin and embedded in paraffin. Sections of 5 µm thickness were cut and stained with haematoxylin and eosin for microscopic examination ($\times 125$ and $\times 500$ magnifications).

Statistical analysis

All results were expressed as the mean $\pm$ SEM, with the number of mice/group indicated in each figure. Differences between groups were analysed using one-way ANOVA from GraphPad InStat (Version 3.05 software, San Diego, CA, USA), followed by Bonferroni inter-group comparison tests and the level of significance was defined at $P < 0.05$.

Human studies

Twelve green gram-allergic patients (mean age: 35.7 ± 1.4 years; eight males and four females) were selected from a population of naso-bronchial allergy during a prospective study performed at the Department of Pulmonary Medicine, Chhatrapati Shahuji Maharaj Medical University (CSMMU), Lucknow, India. The selection was carried out on the basis of a convincing clinical history of allergic symptoms after perceived consumption of

green gram, which was confirmed on the basis of positive skin prick test (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 CSMMU, Lucknow.

Skin prick test and patient's sera collection. SPTs were carried out as described previously [14]. Weal diameter equal to that of positive control or more (>3 mm) were considered as marked positive skin reactions. Blood was collected from 12 SPT-positive patients having history of allergy to green gram. Pooled serum from other 12 patients, allergic to dander and insects but not to any food or pollen extracts was used as negative control. Sera were separated from whole blood without any anticoagulant as per as the method of serum separation given at the website http://www.protocolonline.org/cgi-bin/prot/view_cache.cgi?ID=3096 and stored at -20°C .

Total immunoglobulin E estimation. Total serum IgE levels in patients was determined using commercially available double antibody sandwich ELISA kit following the manufacturers' instructions (Immunology Consultants Laboratory Inc., Newberg, OR, USA). IgE concentration was calculated using the standard curve and presented as IU/mL ($1\text{ IU/mL} = 2.4\text{ ng/mL}$).

Specific immunoglobulin E estimation. Specific IgE levels against green gram were estimated by ELISA using the method of Voller et al. [18]. Briefly, the wells of microtitre plate (Nunc) were coated with $1\text{ }\mu\text{g}$ of protein in carbonate buffer (pH 9.6). After blocking, the plates were incubated with diluted sera ($1:10$ v/v) of green gram-hypersensitive patients overnight at 4°C . The plate was washed and incubated with HRP-conjugated goat anti-human IgE ($1:1000$ v/v, Sigma Chemical Co.) for 3 h at 37°C . Colour was developed with OPD. The reaction was stopped after 20 min by adding $5\text{ N H}_2\text{SO}_4$ and the absorbance was read at 492 nm.

Identification and characterization of green gram allergens

Immunoglobulin E immunoblotting of green gram crude protein extract. For detecting IgE-binding proteins of green gram, immunoblotting with sera of green gram-hypersensitive patients was performed. SDS-PAGE-resolved proteins were electrophoretically transferred on to a polyvinylidene difluoride (PVDF) membrane with pore size $0.45\text{ }\mu\text{m}$ (Immobilon-P, Millipore Co., Billerica, MA, USA), according to Towbin et al. [21], for 2 h at 0.8 mA/cm^2 . After termination of the protein transfer, the membrane was blocked with 5% defatted milk in PBS-T buffer and kept overnight at 4°C . After three washings (5 min each) with PBS-T, the strips were incubated with

sera of allergic patients overnight at 4°C [dilution $1:20$ in PBS-T+3% bovine serum albumin (BSA)]. After three washes again, the membranes were further incubated for 6 h at RT with goat anti-human IgE peroxidase conjugate (Sigma Chemical Co., $1:1000$). IgE-binding protein bands were detected using enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Amersham, UK), according to the manufacturers' protocol. Images were captured using a Syngene gel documentation system equipped with CCD camera (Syngene).

Two-dimensional electrophoresis and immunoglobulin E immunoblotting of green gram proteins. Green gram CPE ($100\text{ }\mu\text{g}$) in rehydration buffer was resolved over a linear pH 3–10 immobilized pH gradient (IPG) strip (GE Healthcare Life Sciences, Piscataway, NJ, USA) in first dimension. Strips were focused up to $20\text{ }000\text{ VhT}$, with a maximum of 4500 V , at 20°C using an Ettan IPGPhor 3 (GE Healthcare Life Sciences). Second-dimension separation was performed over 12% SDS-PAGE gels. Identical gels were stained with CBB G-250 or blotted on PVDF membrane and incubated with pooled patients sera ($1:20$ in PBS-T with 3% BSA), followed by HRP-conjugated goat anti-human IgE antibody (Sigma Chemical Co.). Two-dimensionally separated green gram CPE were also immunoblotted with pooled sera of green gram-sensitized mice ($1:20$ in PBS-T with 3% BSA) and probed by HRP-conjugated rat anti-mouse IgE antibody (Southern Biotech, Birmingham, AL, USA). IgG from patients as well as mice sera were removed using protein G-agarose beads before immunoblotting (Pierce Biotechnology, Rockford, IL, USA).

Liquid chromatography mass spectrometry analysis of immunoglobulin E immune-reactive proteins. CBB G-250-stained gel and immunoblot were matched with the help of the two-dimensional (2D) platinum software (GE Healthcare Life Sciences). Four proteins (52, 50, 30 and 18 kDa) showing IgE binding with both green gram-allergic patient's sera as well as sensitized BALB/c mice sera were excised carefully from 2D gel and subjected to liquid chromatography mass spectrometry (LC-MS/MS) at The Centre for Genomic Applications (TCGA), New Delhi. 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. MOWSE scores >45 were considered as significant.

Sequence analysis. A high probability-based MOWSE score was used to identify proteins. To decipher the order of resultant tryptic-digested peptides in the probable sequence, alignment of individual peptide sequence was run with the protein hit having maximum probability score using CLUSTAL W program on Biology Workbench 3.2 [22]. The resulting ordered peptide sequence was subjected to overall FASTA search on www.allergenonline.com version

9.0 to determine allergenicity of proteins by showing significant homology (e -score <0.001 was taken as cut-off) and any value less than this was considered significant for sequence matching.

Results

Simulated gastric fluid digestibility of green gram crude protein extract

SDS-PAGE of green gram CPE accrued in eighteen proteins ranging from 18 to 170 kDa (Fig. 1, lane 2). Out of 18, three proteins of 52, 35 and 30 kDa were stable up to 60 min (Fig. 1, lane 11), whereas two proteins of 18 and 38 kDa proteins were digested after 2 and 15 min, respectively (Fig. 1, lanes 7 and 10), following SGF digestion. A new protein band of 20 kDa appeared after 15 s (Fig. 1, lane 5) and it remained undigested up to 60 min in SGF (Fig. 1, lane 11).

Animal studies

Antigen challenge of sensitized mice resulted in anaphylactic symptoms and increased histamine levels. To quantify the extent of anaphylactic responses induced by green gram proteins, allergic hypersensitivity symptoms were observed in sensitized mice after challenge. Hypersensitive symptoms became evident within 15–30 min after challenge. Mice sensitized with green gram showed severe anaphylactic symptoms with 60% mortality (Fig. 2a), whereas mortality rate was 80% in case of peanut CPE (positive control)-treated group. However, negative con-

trol mice showed no symptoms of hypersensitivity after green gram or peanut challenge. In accordance with the anaphylactic symptoms, histamine level in plasma of green gram-sensitized mice increased sevenfold after allergen challenge as compared with controls (Fig. 2b). These results advocate green gram CPEs ability to induce severe allergic symptoms in sensitized mice.

Specific immunoglobulin E and G1 levels markedly increased in mice sera sensitized to green gram. The level of specific IgE and specific IgG1 was observed to be significantly higher ($P < 0.001$) in green gram as compared with the vehicle-treated group (Figs 2c and d). The elevation of specific IgE was more on day 43 and did not diminish by the 59th day of treatment (Fig. 2c). Green gram-sensitized group also showed a significant increase in IgG1 levels ($P < 0.001$) up to 59 days (Fig. 2d).

Green gram proteins induced T helper type 2 cytokines at messenger ribonucleic acid and protein levels. The m-RNA levels of IL-4 and IL-5 were up-regulated approximately twofold, whereas IL-10 was up-regulated less than two-fold in spleen and threefold in blood of green gram-sensitized mice (Figs 3a and b). Likewise, 1.5–3.0-fold increase was found in IL-4 and IL-10 levels in splenocyte supernatants at all time-points tested (Figs 3c and d). Enhancement in Th2 cytokines at mRNA as well as protein levels was highly significant ($P < 0.01$) over the control.

Histopathology of lungs and spleen showed pathological changes indicative of allergy. To study the allergenic effect of green gram at tissue level, histopathology studies were performed for both lungs and spleen. Lung histopathology of green gram-challenged mice showed perivascular and peribronchial inflammatory cell infiltrate with mild narrowing of the bronchiolar lumen. Thickening of alveolar septa throughout the parenchyma was also observed (Fig. 4c). Mild bronchial epithelial hyperplasia leading to moderate narrowing of bronchiolar lumen was observed in peanut-treated groups (Fig. 4b). Spleen from green gram as well as peanut-challenged group showed lymphoid hyperplasia with activated macrophages. In addition, large megakaryocytes were also evident (Figs 4e and f). Lungs and spleen from control group presented normal histological structure (Figs 4a and d).

Human study

All the 12 selected patients experienced breathlessness, while six patients had itching in mouth and abdominal cramps and four were showing urticaria after green gram consumption. The selected patients were suffering with breathing problems and 50% of these patients were having both allergic rhinitis and bronchial asthma, while 25% patients had allergic rhinitis with urticaria. At

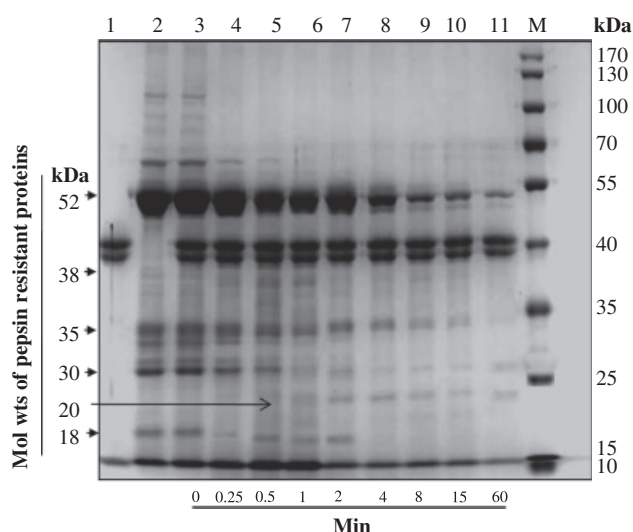


Fig. 1. SDS-PAGE of simulated gastric fluid (SGF) digested green gram crude protein extract (CPE). Lane 1: Pepsin; Lane 2: green gram CPE; Lanes 3–11: SGF digestion pattern of green gram CPE at 0, 0.25, 0.5, 1, 2, 4, 8, 15 and 60 min; Lane M: molecular weight markers.

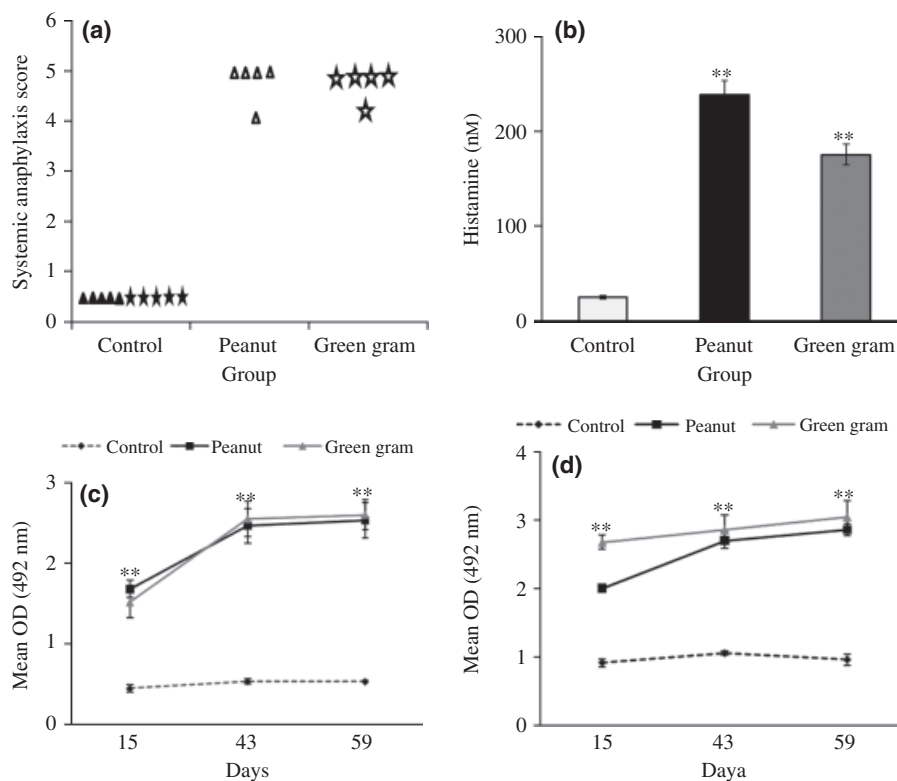


Fig. 2. Parameters demonstrating allergic potential of green gram crude protein extract (CPE). (a) Evaluation of anaphylactic reactions in green gram-sensitized BALB/c mice. Anaphylactic symptom score observed in mice after 30–40 min following green gram CPE challenge. Separate controls for green gram and peanut are indicated by solid stars and solid triangles, respectively. (b) Plasma histamine levels. Thirty minutes following green gram CPE challenge, blood was collected EDTA-coated tubes and plasma histamine levels were measured using an enzyme immunoassay. The samples were run in triplicate and data are expressed as mean $\pm$ SEM for five mice from each group. Significant difference (** $P < 0.001$) compared with the control. (c) Specific IgE and (d) Specific IgG1 in sera of green gram-sensitized mice. BALB/c mice were intraperitoneally administered 100 μ g CPE/100 μ L phosphate-buffered saline (PBS) on days 0, 7, 14, 21, 28, 35 and 42. Blood was collected on days 15, 43 and 59. The samples were run in triplicate with two independent experiments. Data are expressed as mean $\pm$ SEM for five mice from each group. Significant difference (** $P < 0.001$) as compared with vehicle control.

least 50% of patients had gastrointestinal problems. Twelve patients with marked positive SPT (2+ or more) to green gram extracts had total IgE levels ranging from 706 to 3054 IU/mL, whereas non-allergic subjects had total IgE level ranging from 150 to 3042 IU/mL. The levels of specific IgE in patients allergic to green gram were escalated by 5–6.5-fold compared with negative control (patients having allergy to dander and insects). Green gram-allergic patients showed concomitant sensitization to other legumes as evident by marked positive skin reactions elicited by different legumes (Table 1). IgE immunoblot of green gram CPE with pooled patient's sera showed five proteins of 52, 38, 35, 30 and 18 kDa (Fig. 5). The IgE reactivity of green gram CPE was also analysed by individual patient's serum. The 52 kDa protein was recognized by all the patient's sera, while proteins of 30, 35 and 18 kDa were recognized by 67%, 50% and 33% of patients' sera, respectively. Immunoblot with pooled negative control human sera did not show any specific IgE binding.

Identification and characterization of immunoglobulin E-binding proteins of green gram

Sera from green gram-sensitized mice recognized four IgE-binding proteins of 52 kDa ($\sim$ pI 5.7), 50 kDa ($\sim$ pI 5.8), 30 kDa ($\sim$ pI 6.6) and 18 kDa ($\sim$ pI 5.5) (Fig. 6b). Pooled sera of 12 patients showed six IgE-binding protein spots of 52, 50, 38, 35, 30 and 18 kDa (Fig. 6c). Common proteins showing IgE binding to both mice and human sera were 52, 50, 30 and 18 kDa. These were tentatively named as Vig r2, Vig r3, Vig r4 and Vig r5, respectively (as Vig r1 has been reported in mung bean seedling, [15]).

On the basis of the significantly high MOWSE score, mass spectrometric analysis revealed Vig r2, Vig r3 and Vig r4 protein spots as 8S globulin β -isoform precursor, 8S globulin α -isoform precursor and seed albumin of green gram, respectively (Table 2). However, mass spectrometric analysis of Vig r5 did not show high MOWSE score to any green gram proteins.

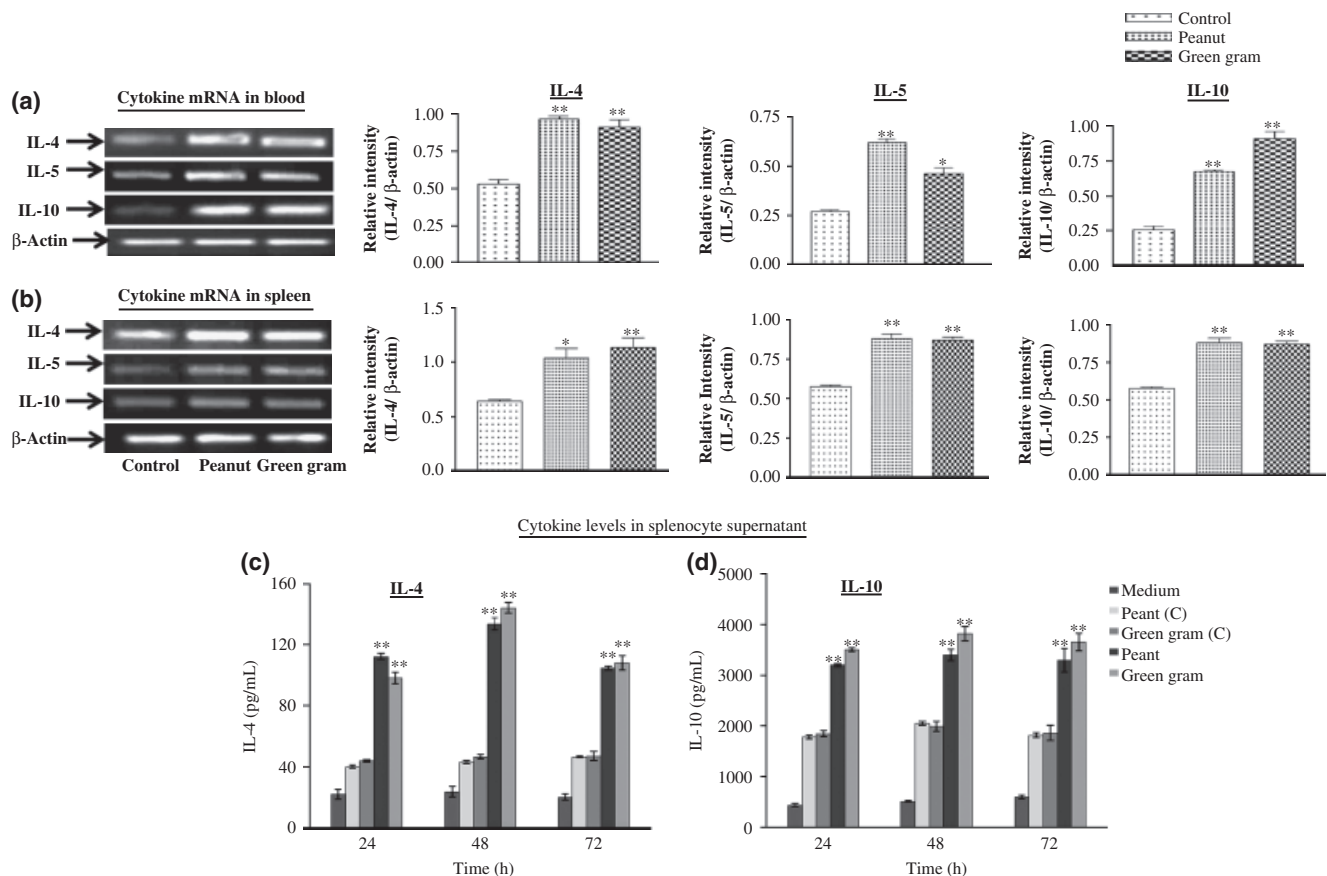


Fig. 3. Green gram challenge enhances Th2 cytokines at mRNA (a–b) and protein levels (c–d). mRNA of Th2 cytokines included IL-4, IL-5 and IL-10 in whole blood (a) and spleen (b). β -actin was taken as an endogenous control and its respective densitometric values were used for normalizing different cytokines in blood and spleen separately. Normalized mRNA cytokine values were used for plotting the bar graphs. RT-PCR results representative of two separate reproducible experiments are expressed as the mean $\pm$ SEM. Levels of IL-4 (c) and IL-10 (d) were measured in splenocyte culture by ELISA. The data from at least two independent set of experiments are expressed as the mean $\pm$ SEM. * $P < 0.01$ and ** $P < 0.001$ are green gram-sensitized and primed mice vs. green gram primed naive mice.

Sequence analysis

Ordered tryptic fragment sequence of all these protein spots were arranged with CLUSTAL W and subjected to BLAST search against NCBI nr protein database. 8S globulin β -isoform precursor (Accession # ABG02262.1), 8S globulin α -isoform precursor (Accession # ABW23574.1) and seed albumin (Accession # CAA50008.1) proteins of green gram were not identified as allergens before this study, as these proteins of green gram are not included in any of the allergenic database like AllergenOnline, SDAP, IUIS and Allergome. To assess IgE-binding protein's sequence similarity to other allergens, their amino acid sequences were subjected to over all FASTA search on AllergenOnline database. Upon full-length FASTA search, where identity matches $> 50\%$ indicate possible cross-reactivity, 8S globulin β -isoform precursor and 8S globulin α -isoform precursor showed homology to well-known allergenic proteins from food crops, 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 conglutin β of lupin. 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 contain two cupin-conserved domains and are members of cupin superfamily (supporting information Fig. S1).

Discussion

There is a dearth of erudition about allergic responses caused by green gram and its clinically relevant allergens. Our previous finding indicated that many individuals having naso-bronchial allergy were co-sensitized to green gram as well as soybean [14]. Therefore, the present study was undertaken to evaluate the allergenic potential of green gram in animal model, if any, pursued by characterization of allergens present therein using green gram-sensitized mice and allergic patient's sera.

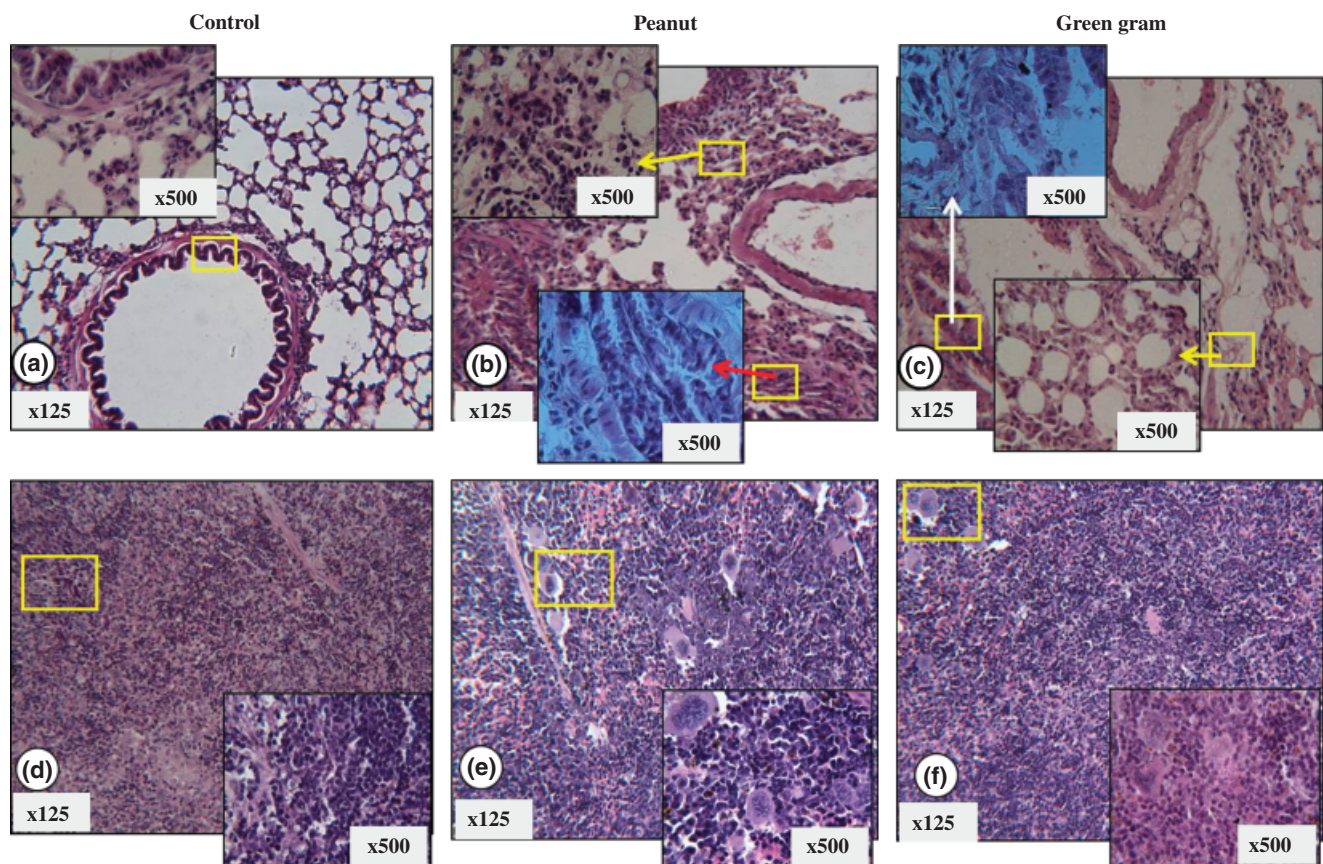


Fig. 4. Histopathological responses induced by green gram administration in lung and spleen. Lung histology of sensitized/challenged mice: (a) Control, (b) Peanut and (c) Green gram. Perivascular and peribronchial inflammatory cell infiltrate is indicated with yellow arrow. Narrowing of the bronchiolar lumen in green gram-treated group indicated with white arrows. Mild bronchial epithelial hyperplasia was evident in peanut-treated groups (indicated with red arrow). Spleen histology of sensitized/challenged mice: (d) Control, (e) Peanut and (f) Green gram. Lymphoid hyperplasia and large-sized megakaryocytes are indicated in inset.

Table 1. Clinical and immunological analysis of green gram-sensitive allergic patients

Patient no.	Age (years)/Sex	Symptoms	SPT with green gram CPE	Total IgE (IU/mL)	Specific IgE (OD)	Sensitization to other legumes
1.	33/M	AR/BA	2+	706.39	0.89	Cc, Gm, Ca#
2.	26/M	AR/URT	2+	1288	1.09	Cc, Pv, Lc
3.	42/F	AR/BA/GIS	3+	3054.33	1.80	Gm, Ca,
4.	20/M	AR/BA/URT	2+	1245.90	1.29	Cc, Gm, Ca
5.	35/F	AR/GIS	3+	2029.09	2.03	Cc, Cc, Ca
6.	34/M	BA	2+	848	1.007	Cc, Gm
7.	36/M	AR/GIS	3+	3040	1.03	Gm, Cc
8.	45/F	BA/URT	2+	1142	0.7685	Cc, Gm, Ca
9.	40/M	AR/BA/GIS	2+	2028	1.004	Cc, Lc
10.	33/M	AR/BA/URT	2+	1110	0.82	Cc, Ca, Gm
11.	16/F	BA/GIS	3+	1142	1.39	Gm, Ca
12.	32/M	AR/BA/GIS	2+	725	0.79	Cc

Negative control for specific IgE (sera from dander and insects allergic patients): OD 0.16–0.31.

Negative control for total IgE (sera from non-allergic patients): 150–3042 IU/mL

AR, allergy rhinitis; BA, bronchial asthma; URT, urticaria; GIS, gastrointestinal symptoms; M, male; F, female; SPT, skin prick test; CPE, crude protein extract; Cc, *Cajanus cajan*; Gm, *Glycine max*; Pv, *Phaseolus vulgaris*; Ca#, *Cicer arietinum* (a different cultivar of chickpea); Ln, *Lens culinaris*; Ca, *Cicer arietinum*.

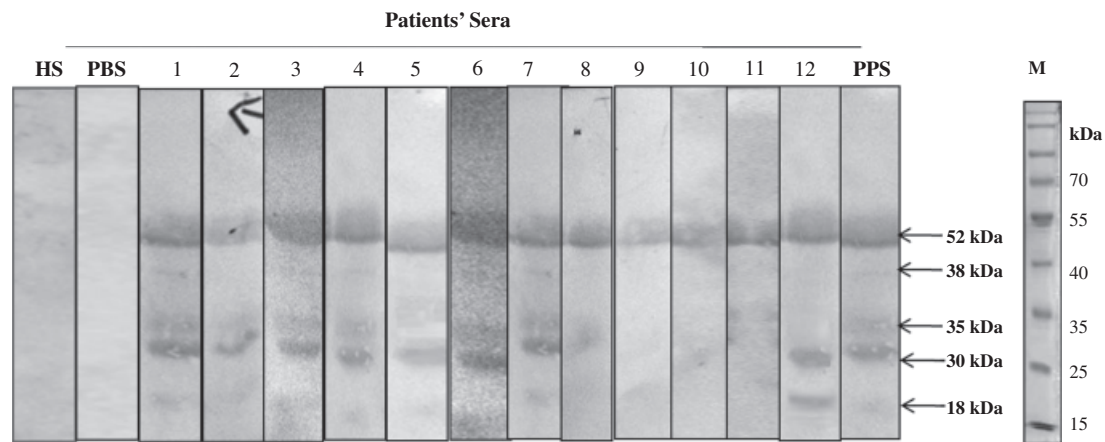


Fig. 5. IgE immunoblots of individual/pooled green gram-sensitive patient sera. Lane HS, human sera (negative control); PBS, green gram crude protein extract (CPE) incubated with PBS and anti-human IgE-HRP (1 : 1000); 1–12 individual green gram-sensitive patient serum of 12 patients (1 : 20); PPS, pooled patients' sera (1 : 20); Lane M, protein molecular weight markers.

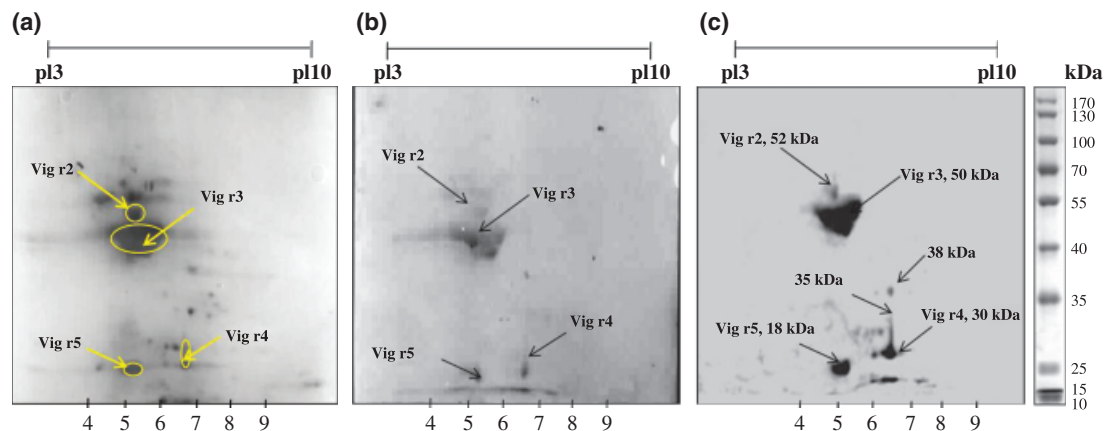


Fig. 6. Two-dimensional (2D) electrophoresis of green gram crude protein extract (CPE) and IgE immunoblotting of 2D resolved proteins. (a) 2D SDS-PAGE profile of green gram CPE. Green gram CPE were separated by isoelectric focusing (IEF) (pH 3–10) followed by SDS-PAGE (12%) and stained thereafter by CBB. (b) IgE-binding proteins detected by immunoblotting of 2D-resolved green gram CPE with pooled sera (1 : 20) of green gram-sensitized mice. Horseradish peroxidase (HRP)-labelled secondary antibody (1 : 1000) directed to mouse IgE was used to visualize spots by enhanced chemiluminescence (ECL). (c) IgE-binding proteins detected by immunoblotting of 2D-resolved green gram CPE pooled serum of patients allergic to green gram. Sera from green gram-sensitive patients (1 : 20) were used as primary antibody; anti-human IgE-HRP (1 : 1000) was used to visualize spots by ECL. The encircled proteins corresponding to IgE-binding immunoblots were excised from 2D electrophoresed gel for mass spectrometry.

One of the important characteristics responsible for allergenicity of food proteins is their stability against pepsin digestion [10–12]. The theoretical basis for association of resistance to SGF digestion with allergenicity is that allergens are stable proteins that therefore persist in the gastrointestinal tract in an intact form, allowing the protein or its large peptides to have sufficient opportunity to interact with the intestinal immune system and stimulate it, causing allergic responses. Recently, Adachi et al. [13] have shown difference in resistance to digestion of two soybean-based foods and suggested that the presence of undigested allergens in the digestive tract is a prerequisite for the development of food-dependent exercise-induced anaphylaxis (FDEIA). In an *in vivo* study, Crowe

et al. [23] reported that a pepsin-resistant protein increased the intestinal permeability by 15-fold, thereby enhancing the chances of sensitization of animal to exposed protein. Pepsin resistance in combination with the presence of trypsin inhibitor(s) may be one of the important reasons behind the green gram protein's behaviour as allergens. Our earlier study also indicated that proteins stable in SGF showed an ability to bind with specific IgE of allergic patient's sera [16]. In green gram CPE, five proteins (52, 38, 35, 30 and 18 kDa) were found to be resistant to SGF digestion, indicating that these proteins and/or their smaller peptide products may interact with intestinal immune system for adverse reactions. It can be inferred, therefore, that these SGF-stable

Table 2. Mass spectrometric analysis of green gram IgE-binding proteins

Sl. no.	Tentatively named IgE-binding protein	Tryptic peptide fragments	Sequence similarity with proteins and their accession*	Probability-based MOWSE score (ion score)*	Expected mol. wt. (kDa)	Calculated mol. wt. (kDa)
1.	Vig r2	RVVEFKS KLAIPVNNPHRF RAILTLVNPdGRD KQIQNLENYRV KERGLLLPHYNSKA KDLDVFISSVDMKE KELSSQDEPFNLRN RWYEITPEKNPQLKD	8S globulin β -isoform precursor (<i>Vigna radiata</i>) (Accession # ABG02262.1)	94	52	51.75
2.	Vig r3	RNQFGHLRV RDLDMFIRS REQUIRELTKH RSKQMQLNENYRV KKSLSSEDQPFNLRN KNILEASFDSIKEISRV RSVDMKEGSLLLPHYNSKA KSLSSDQPFNLRNQKPIYSNKL RNFLAGEKDNVISEIPTVLDVTFPASGEKV	8S globulin α -isoform precursor (<i>Vigna radiata</i>) (Accession # ABW23574.1)	236	50	51.9
3.	Vig r4	RIDYDSKQ KQLVGSIRN KEVYLFKG RIHFTPGKT RLQYTPGKT KGKEVYLFKG RNTVFADSIDSFRS KILAGPTTIAEMFPVLRN	Seed albumin (<i>Vigna radiata</i>) (Accession # CAA50008.1)	148	30	30.5
4.	Vig r5	KTLSSQDKPFNLRN	Canavalin (Sword bean)	60	18	–

*Ions score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. Individual ions scores > 44 indicate identity or extensive homology ($P < 0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

proteins have potential for absorption by the intestinal mucosa and hence enhanced chances of these to behave as allergens.

We observed that green gram pre-sensitized mice on challenge showed visible signs of anaphylaxis. These systemic anaphylactic symptoms involved multiple target organs, including gut and respiratory tract. Histamine augments pathogenesis of allergy process [24] and may be responsible for release of Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13 and inhibits Th1 cytokines IL-2, IFN- γ and IL-12 release [25, 26]. Upon allergen-mediated cross linking of mast cell-bound IgE, histamine and other mediators are released and contribute to the symptoms of anaphylaxis. Data in the present study also reveal that green gram induced manifold increase in plasma histamine levels and severe anaphylactic symptoms in challenged mice, similar to findings of our earlier investigation [19] on another legume red gram.

Food allergy, for the most part, is mediated by allergen-specific IgE antibodies that involve Th2 response. Earlier studies have documented that IgG1 antibodies are associated with Th2 cells [27]. IL-4 enhances IgE and IgG1 (non-complement fixing IgG isotype) production. It was observed

in the current study that green gram administration up-regulated Th2 cytokines including IL-4. This modulation in levels of Th2 cytokines is in coherence with increased IgE and IgG1 levels in sera of green gram-sensitized mice strongly supporting the allergenic potential of green gram and corroborated with our previous finding [19].

Lung sections of green gram-challenged mice revealed build up of inflammatory cells in the perivascular and peribronchial regions of the lung parenchyma with contraction of bronchiolar lumen. Lung histology further confirmed that exposure to green gram increased inflammatory cells and caused airway blockage (Fig. 4c). Airway hyperresponsiveness is mostly observed in immune-inflammatory diseases like asthma and is characterized by peribronchial recruitment of inflammatory cells [28]. The lymphoid hyperplasia observed in our study is a typical histological attribute of the spleens in allergic response (Fig. 4f). It may be because of massive proliferation and differentiation of T lymphocytes, which are main histological signs of chronic inflammation in lymphoid organ [29]. These histological changes support the modulation in cytokines and IgE and IgG1 levels, undoubtedly pinpointing the role of green gram in allergic exacerbations.

Having observed the allergic potential of green gram in BALB/c mice, next we were interested to know its sensitization in human population. Green gram-allergic potential in human was clear from the extent of sensitization in allergic patients and was further confirmed by the presence of specific IgE in human sera. We observed the extent of sensitization to green gram in allergic patients that was further confirmed by the presence of specific IgE in human sera. An earlier study had also unveiled that SPT-positive chickpea patients had enhanced IgE levels, confirming our findings [30]. Our study showed marked concomitant sensitization to other legumes like soybean, chickpea and green gram, which may be due to certain common epitopes bestowing allergenic property to proteins validated with sequence homology and ELISA inhibition studies (supporting information Fig. S2).

SDS-PAGE immunoblot with pooled patient's sera demonstrated five proteins of 52, 38, 35, 30 and 18 kDa having IgE-binding potential. Interestingly, all these five IgE-binding proteins were resistant to SGF digestion. Two-dimensional electrophoresis-resolved electroblot showed six IgE-binding proteins of 52, 50, 38, 35, 30 and 18 kDa when incubated with patient's sera and recognized four proteins of similar molecular weights of 52, 50, 30 and 18 kDa with mice sera. This shows similarities between mice and human immune system and their reagenic antibody responses [31].

Mass spectrometric analysis revealed Vig r2 to be 8S globulin β -isoform precursor, Vig r3 as 8S globulin α -isoform precursor and Vig r4 as seed albumin from green gram with high probability-based MOWSE score. Vig r5 did not show significant similarity with any known green gram protein, although its ordered peptides showed sequence homology to known allergens of other legumes like soybean, lentil, pea and lupin (supporting information Table S1). β - and α -isoform precursors of 8S globulin are major seed storage proteins of green gram and belong to the cupin superfamily, a ubiquitous protein, as cupin-conserved domain is present in amino acid sequence of these two proteins. A number of cupins have been identified as major plant food allergens, including the 7S globulins of soybean (β -conglycinin), peanut (conarachin; Ara h 1), lentil (Len c1), 11S globulins of peanut

(arachin; Ara h 3), etc. [32]. However, this is the first report of the green gram seed proteins belonging to cupin superfamily demonstrating allergenic properties. Sequence matching of Vig r2 and Vig r3 proteins showed significant homology with known allergenic cupin proteins. Sequence homology studies clearly indicate extensive homology to allergen of soybean, peanut, pea, lentil and lupin, thereby supporting evidence of allergenicity observed in mice and human. These observations indicate towards the possibility of sensitization of patients allergic to green gram to above said foods as well.

This is the first study to establish the allergenic potential of green gram proteins and the mechanism involved therein. We have demonstrated conclusively that green gram could induce specific IgE in an animal model that was facilitated by heightened Th2 responses. In addition, at least four novel putative clinically relevant allergens were identified for the first time in green gram seed proteins; of these, 8S globulin β - and α -isoform precursor were confirmed to be members of cupin superfamily.

The identification of allergens or their epitopes could be helpful in diagnosis and also in obtaining hypoallergenic molecules of potential use for immunotherapy using different protein modification techniques. Moreover, green gram transgenic variety (ies) can be generated that may be less allergic than the native crop available in the market at present. These transgenic varieties may be of immense value to patients sensitive to green gram and other crops having homologous allergens.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Analysis of conserved domain of Vig r3 using Blast search. Vig r3 shares conserved domain with Cupin superfamily.

Figure S2. ELISA inhibition of green gram with extract of green gram (—●—), soyabean (---■---), red gram (---▲---) and chickpea (---◆---) using green gram CPE on solid phase. Pooled sera of Green gram allergic patient were used. Mean ($n \pm 3$) and SEs (bars) are represented.

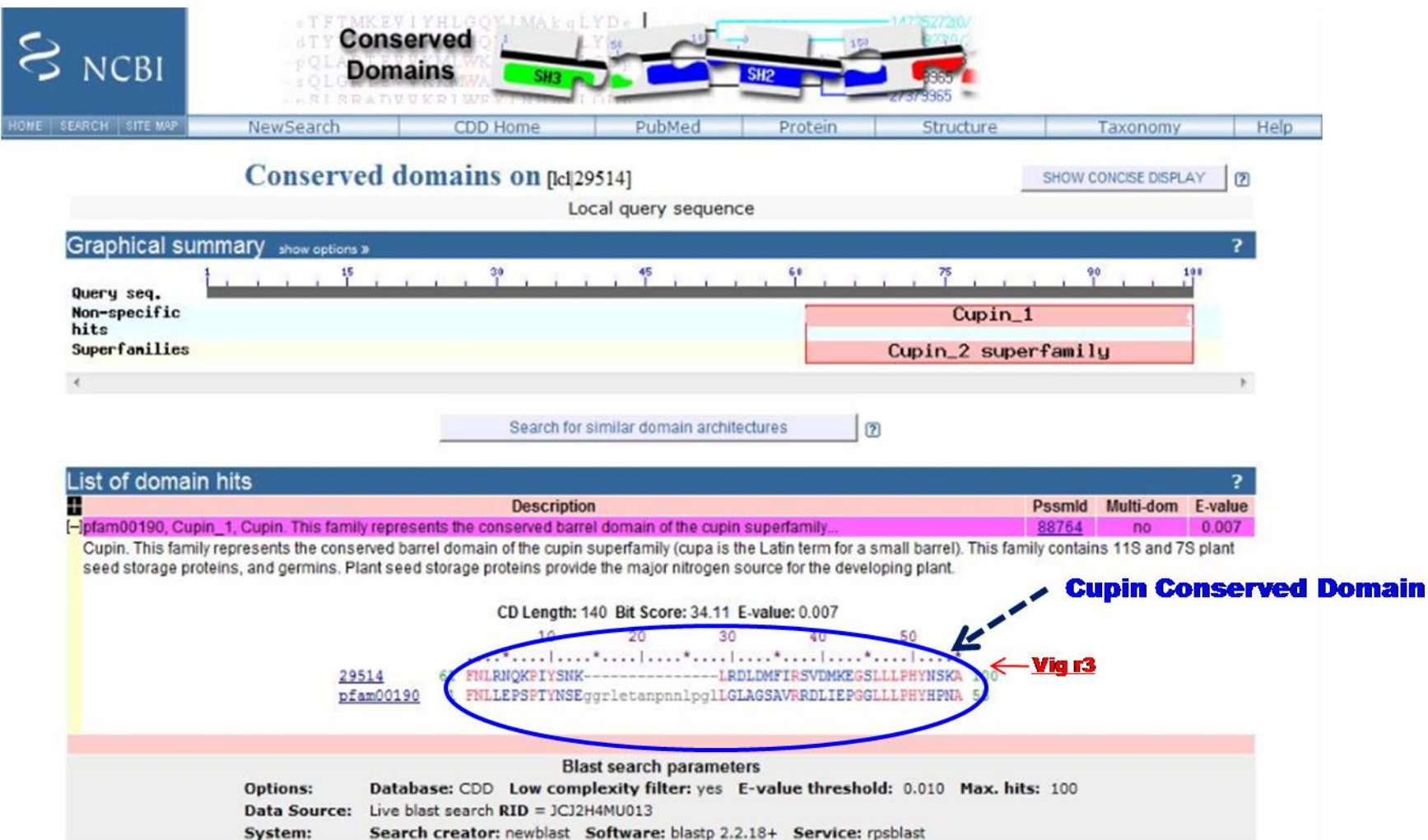
Figure S3. Experimental design. Three groups of BALB/c mice ($n = 15/\text{group}$) were sensitized once a week either with $100 \mu\text{L}$ (1 mg/mL) of peanut or green gram CPE or PBS intraperitoneally. Blood samples were collected on

days 15, 43 and 59. A subgroup of these mice ($n = 5/\text{group}$) and their blood/spleen collected for RT-PCR analysis after challenge with 10 mg of CPE for cytokines mRNA. On day 60, remaining ($n = 5/\text{group}$) mice were challenged and symptoms were scored. Lung and spleen tissues were collected for histopathology. *Blood sampling for specific IgE, IgG1 and sera for 2DE immunoblot (day 43).

Table S1. Sequence homology analysis of tryptic fragments of IgE-binding green gram proteins.

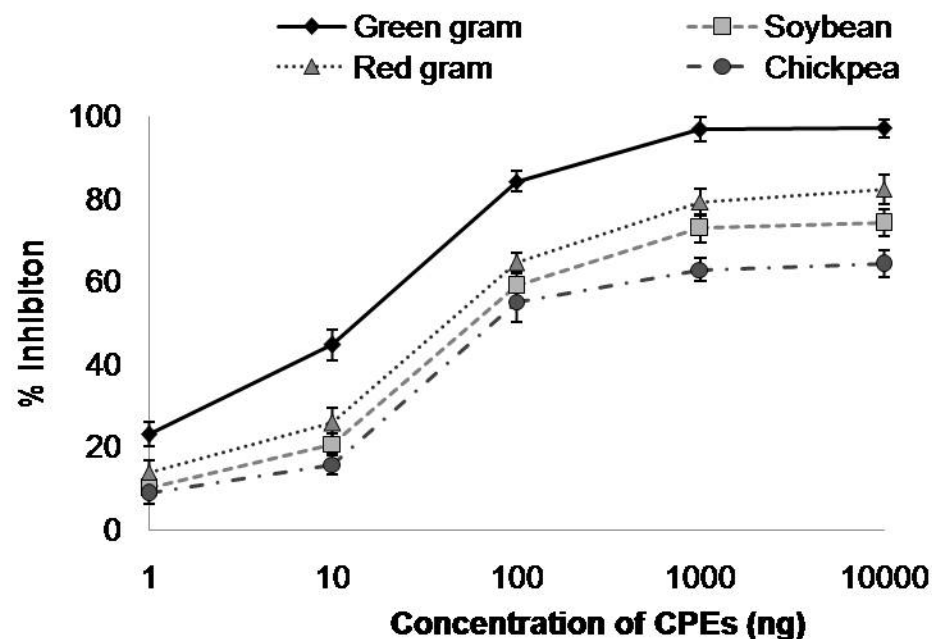
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Fig E1



Analysis of conserved domain of Vig r3 using Blast search. Vig r3 shares conserved domain with Cupin superfamily.

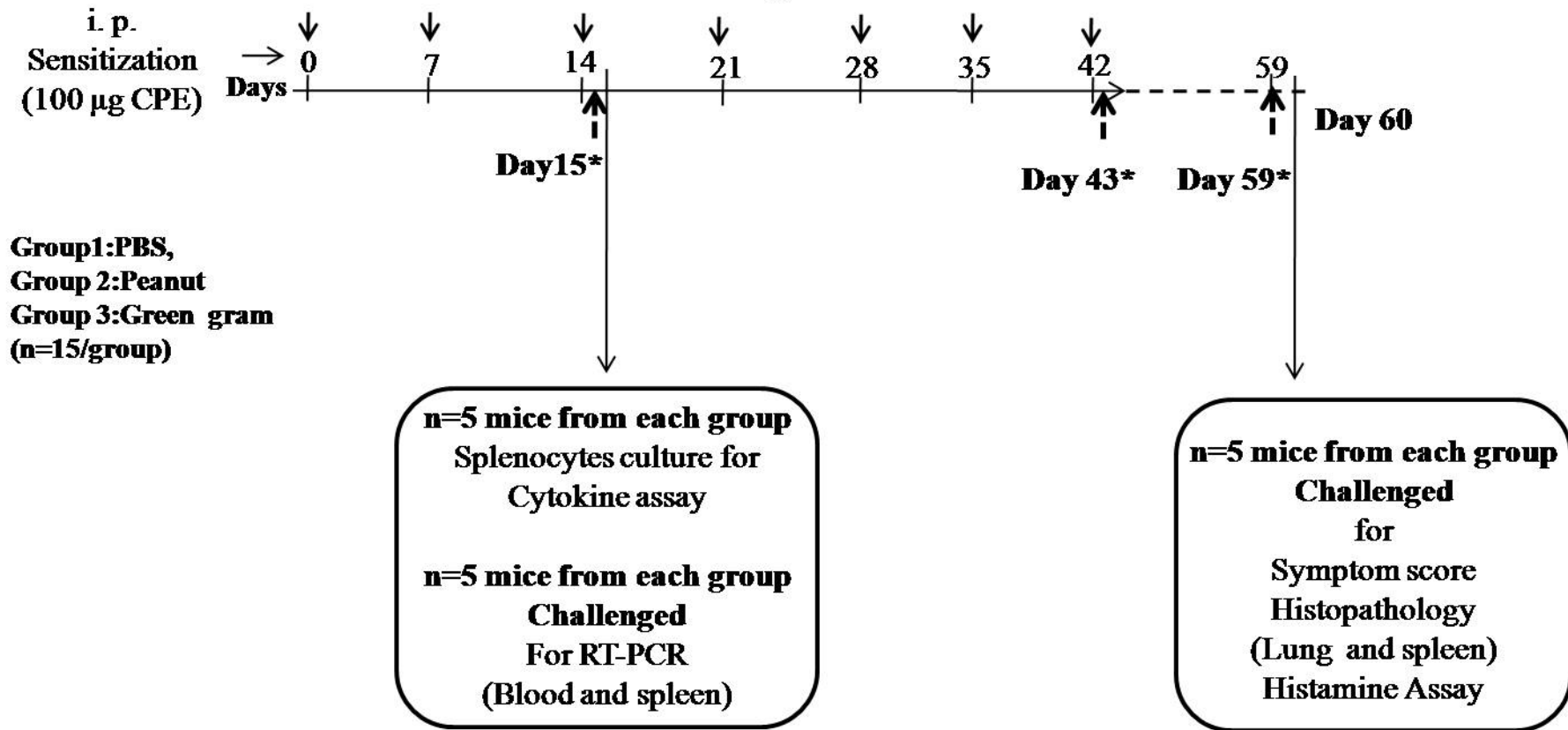
Fig E2



ELISA inhibition of green gram with extracts of green gram (—◆—), soybean (---□---), red gram (···▲···) and chickpea (-●-) using green gram CPE on solid phase.

Pooled sera of Green gram allergic patient were used. Mean (n=3) and SEs (bars) are represented.

Fig E3



Experimental design. Three groups of BALB/c mice (n=15/group) were sensitized once a week either with 100 µl (1mg/ml) peanut or green gram CPE or PBS intraperitoneally. Blood samples were collected on days 15, 43 and 59. A subgroup of these mice (n=5/group) were sacrificed on day 15 to collect spleen for splenocyte culture. Another subgroup of animals sacrificed (n=5/group) and their blood/spleen collected for RT-PCR analysis after challenge with 10 mg CPE for cytokines mRNA. On day 60, remaining (n=5/group) mice were challenged and symptoms were scored. Lung and spleen tissues were collected for histopathology.

***Blood sampling for specific IgE, IgG1 and sera for 2DE immunoblot (day 43)**

Table E1: Sequence homology analysis of tryptic fragments of IgE binding green gram proteins

S. No.	Protein	Tryptic Peptide Fragments	Ordered tryptic fragments	Sequence similarity using Full FASTA search	% identity with ordered fragment (% identity with full protein sequence)
1.	Vig r2 (Accession # ABG02262.1)	KLAIPVNNPHRF	KQIQNLENYRVVE FKSRAILTLVNP GRDKLAIPVNNPH RFKELSSQDEPFNL RNRWYEITPEKNP QLKDLDVFISSVD MKERGLLPHYNS KA	β -conglycinin- α subunit (<i>Glycine max</i>)	57 (68)
		RAILTLVNP RD		β -conglycinin storage protein (<i>Glycine max</i>)	60 (66)
		KQIQNLENYRV VEFKS		Len c 1.0101 (<i>Lens culinaris</i>)	56 (55)
		KEGGLLLPHY NSKA		Len c 1.0102 (<i>Lens culinaris</i>)	56 (55)
		KDLDVFISSVD MKE		Vicilin (<i>Pisum sativum</i>)	40 (55)
		KELSSQDEPFNL RN		Conglutin β (<i>Lupinus angustifolius</i>)	35 (56)
		RWYEITPEKNP QLKD		Ara h1(<i>Arachis hypogaea</i>)	44 (50)
2.	Vig r3 (Accession # ABW23574.1)	RNQFGHLRV	RNQFGHLRVRSK QMQLNENYRVK NILEASFDSDIKEI SRVREQUIRELTKH KKSLSSEDQPFNL RNQKPIYSNKL DLDMFIRSVDMK EGSLLLPYNSK ARNFLAGEKDNV ISEIPTVLDVTFP ASGEKV	β -conglycinin- α subunit (<i>Glycine max</i>)	50 (69)
		REQUIRELTKH		β -conglycinin storage protein (<i>Glycine max</i>)	40 (66)
		RSKQMQLNEN YRV		Len c 1.0101 (<i>Lens culinaris</i>)	45 (57)
		KNILEASFDSDI KEISRV		Len c 1.0102 (<i>Lens culinaris</i>)	54 (57)
		RDLDMFIRSV MKEGSLLLPHY NSKA		Vicilin (<i>Pisum sativum</i>)	56 (57)
		KKSLSSEDQPF NLRNQKPIYSN KL		Conglutin β (<i>Lupinus angustifolius</i>)	48 (58)
		RNFLAGEKDNV ISEIPTVLDVTF PASGEKV		Ara h1(<i>Arachis hypogaea</i>)	39 (51)
3.	Vig r4 (Accession # CAA50008.1)	RLQYTPGKT	RLQYTPGKTKIL AGPTTIAEMFPVL RNTVFADSIDS AFRS RKGKEVYLFKG RIDYDSKQLVGS IRNRIHFTPGKT	-----	-----
		KILAGPTTIAEM FPVLRN		-----	-----
		RNTVFADSIDS AFRS		-----	-----
		RKGKEVYLFKG		-----	-----
		RIDYDSKQLVG SIRN		-----	-----
		RIHFTPGKT		-----	-----
4.	Vig r5 (Accession # ABG02262)	KTLSSQDKPFN LRS	-----	β -conglycinin- α subunit (<i>Glycine max</i>)	79
				β -conglycinin storage protein (<i>Glycine max</i>)	79
				Len c 1.0101(<i>Lens culinaris</i>)	64
				Len c 1.0102 (<i>Lens culinaris</i>)	64
				Vicilin (<i>Pisum sativum</i>)	64
				Conglutin β (<i>Lupinus angustifolius</i>)	70