Credentials
CREDENTIALS

Publications


- Dolly Kumari, Naveen Arora, **Ramkrashan Kasera**, S. Sridhara, Raj Kumar, B.P. Singh. Isolation and characterization of a 28 kDa major allergen from blackgram (*Phaseolus mungo*). *Immunobiology* 2012; 217:895-904.


Poster Presentations


Theoretically, all food products that contain protein may cause allergy and asthma morbidity (8,9). Teenagers and young adults appear to be at higher risk for fatal food allergies. The most frequently reported allergic foods are cow’s milk, hen’s egg, fish, seafood, legumes, wheat and additives (1,10,11). In India, the knowledge about food hypersensitivity is limited to a few clinic-immunological studies. This shows evidence of allergy to foods such as egg, milk, chickpea, rice and black gram in children and adult population (12–15).

Immunoglobulin E (IgE) mediated allergic reactions to legumes like lentil (Lens culinaris) has been reported from Mediterranean paediatric patients (16). Raised specific IgE (sIgE) was demonstrated to be crude and boiled lentil proteins in sera of lentil sensitive children (17). Inclusion and characterization of relevant allergens was also performed from boiled lentil (18). Sensitization and cross reactivity among some legumes are also observed (9–11). Sensitization and cross reactivity of a food allergen may be due to the structural similarity of food proteins and other allergens belonging to different food groups (12–14). The prevalence of IgE-mediated allergy to legumes among the general population is not well known (9,15).

Kidney Bean: A Major Sensitizer among Legumes in Asthma and Rhinitis Patients from India

Ramkrishan Kasera,1,2 Bhavan Pratap Singh,1,3 Sakuntala Lavasa,4 Komera N. Prasad,4 Ramesh C. Sahoo,2 Anand B. Singh1

1 Allergy and Immunology Section, Institute of Genetics and Integrative Biology, Delhi, India, 2University of Pune, Ganeshkhind, Pune, India, 3Lavasa Medical Research Center, Chandigarh, India, 4Bangalore Allergy Centre, Bangalore, India, 5Manipal Medical College, Mangalore, India

Abstract

Background The prevalence of IgE mediated food allergies has increased over the last two decades. Food allergy has been reported to be fatal in highly sensitive individuals. Legumes are important food allergens but their prevalence may vary among different populations. The present study identifies sensitization to common legumes among Indian population, characterizes allergens of kidney bean and establishes its cross reactivity with other legumes.

Methodology Patients (n = 355) with history of legume allergy were skin prick tested (SPT) with 10 legumes. Specific IgE (sIgE) and total IgE were estimated in sera by enzyme-linked immunosorbent assay. Characterization of kidney bean allergens and their cross reactivity was investigated by immunobiochemical methods. Identification of major allergens of kidney bean was carried out by mass spectrometry.

Principal Findings Kidney bean exhibited sensitization in 78 (22.0%) patients followed by chickpea 65 (18.0%) and peanut 53 (15.0%). SPT positive patients depicted significantly elevated sIgE levels against different legumes (r = 0.85, p < 0.0001). Sera from 30 kidney bean sensitive individuals exhibited basophil histamine release (16–54%) which significantly correlated with their SPT (r = 0.83, p < 0.0001) and sIgE (r = 0.99, p < 0.0001). Kidney bean showed eight major allergens of 58, 50, 45, 42, 40, 37, 34 and 18 kDa on immunoblot and required 67.3 ± 2.51 ng of homologous protein for 50% IgE inhibition. Inhibition of IgE binding was also performed from boiled lentil (18). Sensitizati...
severe systemic reactions to red kidney beans (Phaseolus vulgaris) are recorded in some cases [19,20]. Further, studies have proven allergies to chickpeas and black gram in asthma and rhinitis patients and characterized their allergens [13-18]. India is one of the major consumers of legumes since these are an important source of dietary proteins for a large population. However, studies on allergic sensitization to these legumes are confined to few types and the diagnosis is based on various in vitro and in vivo methods in vivo (SPT) and double blind placebo controlled food challenge (DBPCFC) as the gold standard [4,14,15].

The present study was aimed to determine sensitization pattern against commonly consumed legumes in India and to find a correlation among in vitro (SPT) and in vivo (SPT, use IgE and basophil histamine release tests), which may provide quick and easier diagnostic tool, obviating the difficulties associated with DBPCFC.

In addition, IgE binding components of kidney bean (a major sensitizer) and its cross reactivity with other legumes was investigated by immunobiochemical methods using hypersensitive patient’s sera.

Methods

Ethics statement

The present study protocol was approved by the human ethics committee of Institute of Genomics and Integrative Biology, Delhi. Informed written consents was obtained from patients and non-Allergic volunteers for participation in the study.

Study subjects

The study included allergic rhinitis and asthma patients (n = 335) with mean age 30.7 ± 13.9 and history of legume allergy from the two centres, Bangalore (Allergy Centre, Bangalore [n = 198] and L.S.Slavacoo Medical and Research Centre, Chandigarh [n = 137], India. Both the clinical centres are located in geographically distinct places in India in south and north respectively. The food habits also varied among two groups of patients. Of the total, 279 (83.8%) patients were suffering from rhinitis, 11 (3.5%) with asthma and 65 (18.0%) were suffering from both. Interestingly no case of asthma alone was diagnosed from Bangalore centre (Table 1). The diagnosis of asthma and rhinitis was ascertained following American Thoracic Society guidelines, 1991 and Allergic Rhinitis and its Impact on Asthma guidelines, 2001 [21,22]. Patients reporting symptoms such as anaphylaxis, redness of mouth, urticaria, nausea, vomiting, diarrhea, abdominal cramps, running nose or breathlessness after ingestion of legumes were included in the study. Skin prick tests (SPT) were performed with common legume extracts (1:10 w/v) along with a panel of inhalant allergen extracts (procured commercially) from AeroAllergens (UK). The SPT reactions were observed after 20 minutes and wheal diameter ≥ 3 mm were considered positive and graded from 1+ to 4+ based on wheal diameter [23].

Preparation of extracts

Healthy seeds of selected legumes (Table 2) were crushed and defatted in diethyl ether at 4°C. The extraction was carried out following the protocol by Kummari et al. [14]. Extraction of legume allergens after boiling for different time and temperature (15, 30, 45, 60 minutes at 100°C and 15 minutes at 121°C) was also done along with the raw legumes. Protein contents of extracts was determined by modified Lowry’s method [24].

Patients’ sera

Blood was collected from 208 patients with a history of food allergy and SPT positivity to the respective legume(s). Blood was also collected from 20 healthy non-allergic individuals (controls) with negative skin reactivity to different allergens extract. Cases and controls were both sex and age matched.

Table 1. Demographic details of patients skin prick tested with different allergen extracts.

<table>
<thead>
<tr>
<th>Allergen</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruits</td>
<td>162(45.5%)</td>
<td>146(39.4%)</td>
</tr>
<tr>
<td>Foods</td>
<td>154(40.5%)</td>
<td>140(36.2%)</td>
</tr>
<tr>
<td>Pollen</td>
<td>107(28.0%)</td>
<td>92(24.2%)</td>
</tr>
<tr>
<td>Fungus</td>
<td>72(18.5%)</td>
<td>60(16.0%)</td>
</tr>
<tr>
<td>Insect</td>
<td>132(34.0%)</td>
<td>112(30.4%)</td>
</tr>
<tr>
<td>AR + BA</td>
<td>48(12.5%)</td>
<td>40(10.8%)</td>
</tr>
</tbody>
</table>

Table 2. Allergen extracts used in the study.

<table>
<thead>
<tr>
<th>Allergen</th>
<th>% Sensitization (SPT use)</th>
<th>Blood</th>
<th>Protein (μg/ml)</th>
<th>Protein (ng-1 mg)</th>
<th>IgE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furniture</td>
<td>10(2.5%)</td>
<td>80(20.0%)</td>
<td>92(24.5%)</td>
<td>192(51.4%)</td>
<td>34(9.0%)</td>
</tr>
<tr>
<td>Fungi</td>
<td>11(2.8%)</td>
<td>88(22.5%)</td>
<td>90(24.0%)</td>
<td>190(51.2%)</td>
<td>35(9.5%)</td>
</tr>
<tr>
<td>Food</td>
<td>10(2.5%)</td>
<td>80(20.0%)</td>
<td>92(24.5%)</td>
<td>192(51.4%)</td>
<td>34(9.0%)</td>
</tr>
<tr>
<td>Fruits</td>
<td>162(45.5%)</td>
<td>146(39.4%)</td>
<td>154(40.5%)</td>
<td>140(36.2%)</td>
<td>107(28.0%)</td>
</tr>
<tr>
<td>Fungi</td>
<td>72(18.5%)</td>
<td>60(16.0%)</td>
<td>132(34.0%)</td>
<td>112(30.4%)</td>
<td>48(12.5%)</td>
</tr>
<tr>
<td>Furniture</td>
<td>48(12.5%)</td>
<td>40(10.8%)</td>
<td>10(2.5%)</td>
<td>80(20.0%)</td>
<td>11(2.8%)</td>
</tr>
<tr>
<td>Fruits</td>
<td>162(45.5%)</td>
<td>146(39.4%)</td>
<td>154(40.5%)</td>
<td>140(36.2%)</td>
<td>107(28.0%)</td>
</tr>
<tr>
<td>Fungi</td>
<td>72(18.5%)</td>
<td>60(16.0%)</td>
<td>132(34.0%)</td>
<td>112(30.4%)</td>
<td>48(12.5%)</td>
</tr>
<tr>
<td>Furniture</td>
<td>48(12.5%)</td>
<td>40(10.8%)</td>
<td>10(2.5%)</td>
<td>80(20.0%)</td>
<td>11(2.8%)</td>
</tr>
<tr>
<td>Fruits</td>
<td>162(45.5%)</td>
<td>146(39.4%)</td>
<td>154(40.5%)</td>
<td>140(36.2%)</td>
<td>107(28.0%)</td>
</tr>
<tr>
<td>Fungi</td>
<td>72(18.5%)</td>
<td>60(16.0%)</td>
<td>132(34.0%)</td>
<td>112(30.4%)</td>
<td>48(12.5%)</td>
</tr>
</tbody>
</table>

Estimation of Specific IgE

The levels of IgE in SPT positive patients’ sera was determined by ELISA following the protocol of Singh et al. [25].

Estimation of Total serum IgE

Total serum IgE was estimated in sera of patients using kit procured from Bio-Rad Laboratories (USA) as described earlier [26]. IgE values (1 IU/ml = 2.4 mg/ml) were calculated using the standard curve.

Stripped Basophil histamine release assay

Histamine release assay was performed in 30 kidney bean sensitive individuals having significantly high IgE values following the protocol by Kukreja et al. [27]. In brief peripheral blood was drawn from nonallergic donors and mixed with 1:5 v/v with 6% dextran in saline containing 0.01 M EDTA and 2% dextrose. After 90 min, the leukocyte-rich upper layer was drawn, centrifuged and washed twice with saline. The basophils in suspension were stripped off Eppendorf tubes by incubation with lactic acid buffer for 3.5 min [28]. Following incubation, cells were washed in HEPES buffer. Subsequently, the cells were resuspended with a sensitization mixture that contains sera with elevated IgE against kidney bean allergens (n = 30). Cells sensitized with nonallergic sera (n = 5) served as control. The histamine release assay was standardized using a graded amount of procain (1 mg/ml) and the proteins concentration inducing optimal histamine release (5 mg) was selected for the assay. After positive sensitization, cells were stimulated with kidney bean protein (5 mg) in HEPES buffer containing 1 mM CaCl₂. After a 1-h incubation, the reaction was stopped by the addition of 0.3% Na₂EDTA. After centrifugation, the cell-free supernatant containing histamine was taken in a fresh Eppendorf tube and mixed with 12% perchloric acid (Sigma). The histamine content...
Protein profile of boiled extracts (20 μg protein per lane) were loaded on a 12% reducing gel was determined by the bromocresol green method, using o-phthalaldehyde (Sigma). Spontaneous histamine release was measured in the supernatant of unstimulated cells. The total histamine content was determined by lysis of cells with 0.1% perchloric acid. The allergen-induced histamine release was calculated as a percent of the total histamine content after correcting for spontaneous release.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot

To study the protein profile, raw and boiled kidney bean extracts (20 μg protein per lane) were loaded on a 12% reducing gel [29]. Protein profile of boiled (30, 45, and 60 min at 100°C) kidney bean was also analyzed by SDS-PAGE. The resolved proteins were stained with Coomassie brilliant blue R-250 (CBB). Kidney bean was also analyzed by SDS-PAGE. The resolved proteins were transferred on to nitrocellulose membrane as described by Towbin et al. [30]. The immunoblot were excised from coomassie stained gel and subjected to in-gel digestion described by O’Cualain et al. [31] with slight modification.

Mass spectrometric analysis

Peptide ions were injected for analysis by al-LC-MS/MS system (Agilent, Palo Alto, CA, USA) using Agilent 1100 NanoLC-1100 system following manufacturer’s instructions. In brief, the samples (6 μL) were concentrated on pre-column (Zorbax 300SB-C18, 150 mm 75 mm, 3.5 mm) and after 5 min, the pre-column was connected with the separating column, and multistep gradient was started. An LC/MSD Trap XCT with a nano-electrospray interface operated in the positive ion mode was used for MS. Ionization was performed with a liquid junction and a noncoated capillary probe. Peptide ions were analyzed by the data-dependent method as follows: full MS scan. The scan sequence consists of 1 full MS scan followed by 4 MS/MS scans of the most abundant ions. Data were analyzed using Agilent Ion trap Analysis Software version 5.2 and proteins were identified by database search against the MASCOT database.

ELISA inhibition

The allergenic potency of kidney bean extract and its cross reactivity with other legumes was determined by ELISA inhibition. Inhibition of IgE binding was assessed with serum pools of kidney bean positive patients’ preincubated with 5, 10, 50, 100, 500, 1000 and 10000 ng of legumes namely kidney bean, black gram, chickpea, lentil, pea, peanut and pigeon pea as inhibitors. The mixture was added to the solid-phase bound raw kidney bean extract in ELISA plate. Here kidney bean positive patients’ pooled sera without inhibitor was taken as a positive control. To check cross-reactive carbohydrate determinants specific inhibition, if any, kidney bean positive patients’ pooled sera was preabsorbed with bromelain (Sigma) and used for ELISA.

Table 2. Sensitization to legume allergens identified by skin prick tests in patients of asthma, rhinitis or both.

<table>
<thead>
<tr>
<th>Legume extract</th>
<th>Bangalore</th>
<th>Chandigarh</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients SPT No.</td>
<td>Patients SPT %</td>
<td>Patients SPT No.</td>
<td>Patients SPT %</td>
</tr>
<tr>
<td>Kidney bean (Phaseolus vulgaris)</td>
<td>198</td>
<td>32 (16.6%)</td>
<td>157</td>
</tr>
<tr>
<td>Chickpea (Cicer arietinum)</td>
<td>198</td>
<td>49 (25.2%)</td>
<td>157</td>
</tr>
<tr>
<td>Pea (Pisum sativum)</td>
<td>198</td>
<td>18 (9.1%)</td>
<td>157</td>
</tr>
<tr>
<td>Lentil (Lens esculentus)</td>
<td>160</td>
<td>34 (21.3%)</td>
<td>157</td>
</tr>
<tr>
<td>Black gram (Vigna mungo)</td>
<td>198</td>
<td>11 (5.5%)</td>
<td>157</td>
</tr>
<tr>
<td>Green gram (Vigna radiata)</td>
<td>198</td>
<td>28 (14.0%)</td>
<td>157</td>
</tr>
<tr>
<td>Soyabean (Glycine max)</td>
<td>198</td>
<td>29 (15.0%)</td>
<td>157</td>
</tr>
<tr>
<td>Pigeon pea (Cajanus cajan)</td>
<td>198</td>
<td>19 (9.5%)</td>
<td>157</td>
</tr>
<tr>
<td>Pea (Pisum sativum)</td>
<td>198</td>
<td>19 (9.5%)</td>
<td>157</td>
</tr>
<tr>
<td>Lentil (Lens esculentus)</td>
<td>198</td>
<td>19 (9.5%)</td>
<td>157</td>
</tr>
<tr>
<td>Cowpea (Vigna unguiculata)</td>
<td>198</td>
<td>5 (2.0%)</td>
<td>157</td>
</tr>
<tr>
<td>Green gram (Vigna radiata)</td>
<td>198</td>
<td>19 (9.5%)</td>
<td>157</td>
</tr>
<tr>
<td>White kidney bean (Pisum sativum)</td>
<td>198</td>
<td>5 (2.0%)</td>
<td>157</td>
</tr>
<tr>
<td>Black gram (Vigna mungo)</td>
<td>198</td>
<td>19 (9.5%)</td>
<td>157</td>
</tr>
<tr>
<td>Green gram (Vigna radiata)</td>
<td>198</td>
<td>19 (9.5%)</td>
<td>157</td>
</tr>
<tr>
<td>Soyabean (Glycine max)</td>
<td>198</td>
<td>5 (2.0%)</td>
<td>157</td>
</tr>
<tr>
<td>Pigeon pea (Cajanus cajan)</td>
<td>198</td>
<td>5 (2.0%)</td>
<td>157</td>
</tr>
</tbody>
</table>

*p-values were calculated using fisher’s exact test.

http://dx.doi.org/10.1371/journal.pone.0027193.t002
ELISA inhibition was also carried out using Curvularia lunala extract as an inhibitor. Percentage inhibition was calculated as described below.

1- OD of sample with inhibitor X 100

2- OD of sample without inhibitor

Immunoblot inhibition

Immunoblot inhibition was performed to establish cross-reactivity of kidney bean with six common legumes. Only kidney bean positive sera from 7 patients (21.0 O.D.) were pooled and preincubated with 500 μg of kidney bean (homologous extract) and legume extracts namely black gram, chickpea, lima, pea, pinto and pigeon pea, separately. Immunoblot was also performed with Curvularia lunala extract and bromelain as inhibitors. The proteins were transferred on to nitrocellulose membrane, strips were cut, blocked and incubated with the preincubated pooled sera, separately. The rest of the procedure was similar to immunoblotting.

Statistical analysis

Values are represented as mean±SD. Correlation analysis was carried out to study the association among SFl" (sIgE), total IgE (sera), separately. The rest of the procedure was similar to immunoblotting.

Results

Sensitization to legume extracts

The prevalence of sensitization (SFl"-ive) to legumes was observed in 59 (56.0%) patients and it varied from 100 (100.0%) at Bangalore to 107 (68.0%) at Chandigarh. Sensitization (SFl"-ive) to kidney bean was in maximum 107 (68.0%) cases (Table 3). The cut off value to define ELISA positive was 0.138 (OD450) i.e. a three times of the normal control. Intensity of SPT reaction has been found to be very well correlated with sIgE (r = 0.03, p<0.0001) but not with the total IgE levels (r= -0.16, p=0.502). Interestingly, during the follow up study, the patients (history, SPT and sIgE positive) showed no symptoms back to normal once the offending food was withdrawn from their diet.

Table 3. Sensitization to legume allergens detected by sIgE estimation in ELISA.

<table>
<thead>
<tr>
<th>Legume extract</th>
<th>SPT patients positive</th>
<th>ELISA positive cases* (% sensitisation)</th>
<th>sIgE (OD450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney bean</td>
<td>78</td>
<td>75 (98.7)</td>
<td>0.195-3.166</td>
</tr>
<tr>
<td>Chickpea</td>
<td>65</td>
<td>7 (1.8)</td>
<td>0.152-0.009</td>
</tr>
<tr>
<td>Peanut</td>
<td>55</td>
<td>14 (26.4)</td>
<td>0.182-1.182</td>
</tr>
<tr>
<td>Pigeon pea</td>
<td>41</td>
<td>16 (38.5)</td>
<td>0.198-0.711</td>
</tr>
<tr>
<td>Black gram</td>
<td>39</td>
<td>15 (38.4)</td>
<td>0.178-1.035</td>
</tr>
<tr>
<td>Green gram</td>
<td>39</td>
<td>50 (77.5)</td>
<td>0.215-3.380</td>
</tr>
<tr>
<td>Soybean</td>
<td>34</td>
<td>14 (41.2)</td>
<td>0.198-0.637</td>
</tr>
<tr>
<td>Pinto</td>
<td>24</td>
<td>7 (28.6)</td>
<td>0.187-0.0471</td>
</tr>
<tr>
<td>Lentil</td>
<td>7</td>
<td>5 (42.8)</td>
<td>0.216-0.399</td>
</tr>
<tr>
<td>Cow pea</td>
<td>6</td>
<td>233.0</td>
<td>0.198-0.373</td>
</tr>
</tbody>
</table>

*Cut off value for sIgE positivity=0.138 OD (3 times of control).

Protein profile of kidney bean extract

Kidney bean contained 71, 62, and 64 mg protein/g of dry powder in three batches of extracts. SDS-PAGE resolved raw kidney bean extract into 22 coomassie stained protein bands of 14 to 150 kDa (Figure 2, lane 1-3). The extract from boiled kidney bean for 15, 30, 45 and 60 min at 100°C resolved into 19,14,9 and 7 bands whereas the extract from boiled kidney bean (15 min at 121°C) separated into 13 bands on SDS-PAGE (Figure 2, lane 4-8). A new band appeared at 40 kDa in the extract boiled for 15 min at 100°C (Figure 2, lane 4).

Allergenic proteins of kidney bean extract

The IgE binding components of extracts prepared from raw and boiled kidney bean were analyzed by western blot with pooled sera of kidney bean-sensitive patients (n=30) showing SFI" reactivity more than 2x and significant raised sIgE (Table 4, Figure 3A). Raw kidney bean extract showed 13 IgE-binding protein of 120, 95, 76, 58, 55, 56, 43, 42, 46, 37, 34, 36, 24, 18 and 16 kDa (Figure 3A, lane 1) whereas boiled kidney bean for 15 min at 121°C showed only 5 IgE-binding protein bands of 58, 55, 37, 24 and 18 kDa (Figure 3A, lane 2). Boiled kidney bean for 15, 30, 45...
and 60 at 100°C showed 7, 6, 5, and 5 allergenic protein bands, respectively (Figure 3A, lane 3–6). The extract showed heterogeneity in the banding pattern with eight major allergens of 58, 50, 77, 0.0%, of patients’ sera. The strips incubated with normal healthy kidney bean-positive patients’ sera did not show any binding. Kidney bean-positive patients’ sera incubated with Carandina lunata (unrelated) extract could not achieve 100% inhibition of IgE binding in EMISA (Figure 4).

### Mass spectrometric analysis

Analysis of 8 major allergens (protein bands) of kidney bean (Figure 3B) and detailed characterization of identified proteins (Table 5) by nLC-MS/MS resulted in identification of 4 proteins, assigned as Alpha-amylase inhibitor precursor from Phaseolus vulgaris, Full = erythroagglutinating phytohemagglutinin; Full = PHA-E, phaseolin from Phaseolus coccineus, assigned as Alpha-amylase inhibitor precursor from Phaseolus vulgaris. The proteins of 26 and 24 kDa were recognized by 45, 42, 40, 37, 34 and 18 kDa recognized by 79.0% of the patients’ sera. The protein extract showed heterogeneity in the banding pattern with eight major allergens of 58, 50, 77.0% of patients’ sera. The strips incubated with normal healthy kidney bean-positive patients’ sera incubated with Carandina lunata (unrelated) extract could not achieve 100% inhibition of IgE binding in EMISA (Figure 4).
Legume Sensitization and Allergens of Kidney Bean

To assess cross reactivity of kidney bean with 6 different legumes namely black gram, chickpea, lentil, pea, peanut and pigeon pea, KLISA inhibition was performed using different serum pools and respective legume extract as inhibitor. Sixty seven ng of kidney bean protein (homologous) was required to obtain 50.0% inhibition of IgE binding in KLISA, whereas both pranini and black gram (heterologues) caused same inhibition to solid phase kidney bean with 85 ng of protein(s) showing extensive cross reactivity (Figure 5). The extracts of pigeon pea, chickpea, lentil and pea produced 50.0% inhibition with 1000, 7500, 7500 and 10000 ng of protein(s), respectively. Preabsorption of pooled patients’ sera with even 10 ng of Curculonia lunata extract resulted in only 8% inhibition to solid phase kidney bean extract. However, bromelain inhibited 38.0% of IgE binding in KLISA at a concentration of 10 μg (Figure 4).

To establish the cross reactivity of kidney bean allergens immunoblot inhibition was performed using kidney bean positive patients’ pooled sera and self/respective legume extract as inhibitor. Electrophoretically transferred kidney bean proteins were incubated with preincubated serum pool. Preabsorption of pooled patients’ sera with 500 μg of kidney bean extract (self) totally abolished its IgE reactivity (Figure 6, lane 1). For cross inhibition, maximum bands of kidney bean proteins were inhibited by peanut followed by pigeon pea, black gram, lentil, chickpea and pea used as inhibitor (Figure 6, lane 2–7). Peanut, pigeon pea and black gram could inhibit IgE binding of 120, 50, 40, 37, 26, 24, 18, and 16 kDa proteins in kidney bean indicating presence of cross reactive components. Reduction in IgE binding of high molecular-weight kidney bean proteins (>70 kDa) was also recorded on preabsorption of sera with each legume extract(s) tested. However, IgE binding of 55, 50, 45 and 42 kDa proteins could not be inhibited by other legume extract(s). Therefore it can be inferred that these proteins are specific allergens of kidney bean. Preabsorbed sera with bromelain and Curculonia lunata did not show any inhibition (Figure 6, lane 8, 9).

---

**Figure 1.** Histamine release assay. Basophils in leukocyte suspension were stripped off the bound IgE. Stripped basophils were sensitized with sera of kidney bean hypersensitive patients. The sensitized basophils were stimulated with kidney bean extract. The Histamine released from 30 patients varied from 16% to 54% while in controls it is less than 6% (A). Scatter plot of the correlation analysis between % histamine release vs SPT (r = 0.83, p < 0.0001) and % histamine release vs total IgE (D) among 30 kidney bean sensitive individuals. Basophil histamine release has been found to be very well correlated with SPT (r = 0.83, p < 0.0001) and sIgE (r = 0.99, p < 0.0001) but not with total IgE (r = -0.13, p = 0.4942).

**Figure 2.** SDS-PAGE - profile of three batches of raw kidney bean extract Kb1, Kb2 and Kb3 (lane 1, 2, 3 respectively), kidney bean boiled for 15 min at 121 °C (lane 4) and boiled at 100°C for 15, 30, 45 and 60 min (lanes 5–8) stained with Coomassie brilliant blue. M: molecular weight markers.

**Figure 3.** SDS-PAGE - profile of three batches of raw kidney bean extract Kb1, Kb2 and Kb3 (lane 1, 2, 3 respectively), kidney bean boiled for 15 min at 121 °C (lane 4) and boiled at 100°C for 15, 30, 45 and 60 min (lanes 5–8) stained with Coomassie brilliant blue. M: molecular weight markers.
Legume Sensitization and Allergens of Kidney Bean

Figure 3. Immunoblot of raw kidney bean (lane 1), kidney bean boiled for 15 min at 121 °C (lane 2) and boiled at 100°C for 15, 30, 45 and 60 min (lanes 3–6) probed with pooled patients’ sera (1:10 v/v). M: molecular weight markers (A). Immunoblot of kidney bean probed with individual patients’ sera (lanes 1–30), pooled patients’ sera (Lane 31), pool of normal human sera (Lane 32), Curvularia lunata extract tested with kidney bean sensitive patients’ pooled sera (Lane 33), M: Molecular weight marker (B).

doi:10.1371/journal.pone.0027193.g003

Discussion

Studies have suggested that patient’s history, skin testing, provocation tests and in vitro assays provide information on sensitization to ofTending rood(s) [14,17,32]. The present study was undertaken to identify sensitization to predominant legumes in a group of allergic rhinitis and asthma patients (physician diagnosed) from India. In addition, IgE binding components of kidney bean and its cross reactivity among 6 common legumes was also investigated.

Orange et al. [33] reported 64.0% prevalence of food allergy in children with atopic dermatitis and asthma or other respiratory diseases. Ibanez et al. [34] found legumes as the 5th most prevalent source of food allergy with chickpea and bean as predominant cause of allergic reaction in Spanish children. Shaikh [35] observed sensitization to legumes in 20.0% patients of Mumbai, India. A study by Gupta et al. [36] in Eastern India accounts 51.0% of adult food allergy with legumes. In the present study, 58.5% of patients showed sensitization to one or more legumc(s) based on history and SPT results. Kidney bean demonstrated sensitization (SPT +ve) in maximum patients 78 (22.0%) followed by chick pea 65 (18.0%), peanut 53 (15.0%), pigeon pea and black gram (11% each). Previously, Kumar et al. [14] observed sensitization to black gram in only 1.7% of patients. Similar to

Table 5. Peptide mass fingerprint database search of selected spots from 1-DE of kidney bean proteins.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Accession no.</th>
<th>Identification</th>
<th>Matches (Ion score &gt;50*)</th>
<th>Sequence Coverage (%)</th>
<th>MASCOT score</th>
<th>Thn mass/pep (Da)</th>
<th>Obs mass (Da)</th>
<th>Proposed function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAH60239</td>
<td>Alpha-amylase inhibitor precursor (Phaseolus coccineus)</td>
<td>170(9)</td>
<td>22</td>
<td>266</td>
<td>27/0.5/17</td>
<td>18</td>
<td>defensive protein</td>
</tr>
<tr>
<td>2</td>
<td>P05088</td>
<td>Full=Phytoagglutinin phytohemagglutinin; AltName: Full=PHA-E lectin [l&gt;haseolus vulgaris]</td>
<td>39(21)</td>
<td>32</td>
<td>593</td>
<td>28/7/15.5</td>
<td>34</td>
<td>defensive protein</td>
</tr>
<tr>
<td>3</td>
<td>CAQ29133</td>
<td>lectin (Phaseolus vulgaris)</td>
<td>19(7)</td>
<td>21</td>
<td>271</td>
<td>28/6/6/0</td>
<td>37</td>
<td>defensive protein</td>
</tr>
<tr>
<td>4</td>
<td>CAQ29181</td>
<td>Lectin (Phaseolus vulgaris)</td>
<td>15(2)</td>
<td>26</td>
<td>494</td>
<td>28/6/0.8</td>
<td>40</td>
<td>storage protein</td>
</tr>
<tr>
<td>5</td>
<td>AAA9634</td>
<td>Phaseolin (Phaseolus vulgaris)</td>
<td>32(19)</td>
<td>32</td>
<td>321</td>
<td>42/5/0.4/2</td>
<td>42</td>
<td>stress tolerant protein</td>
</tr>
<tr>
<td>6</td>
<td>P07319</td>
<td>Full=Phaseolin, alpha-type</td>
<td>34(17)</td>
<td>26</td>
<td>706</td>
<td>42/5/25.25</td>
<td>45</td>
<td>storage protein</td>
</tr>
<tr>
<td>7</td>
<td>AAA26576</td>
<td>Group 3 late embryogenesis abundant protein (Phaseolus vulgaris)</td>
<td>7(1)</td>
<td>16</td>
<td>328</td>
<td>50/5/0.5/0</td>
<td>50</td>
<td>stress tolerant protein</td>
</tr>
<tr>
<td>8</td>
<td>AAA26579</td>
<td>Group 3 late embryogenesis abundant protein (Phaseolus vulgaris)</td>
<td>4(2)</td>
<td>6</td>
<td>130</td>
<td>50/5/0.5</td>
<td>58</td>
<td>stress tolerant protein</td>
</tr>
</tbody>
</table>

Footnotes: Abbreviations; Thn = Theoretical; Obs = Observed; Matches = Number of peptides matched with protein in MS/MS query; Sequence coverage = Total percentage of protein amino acid sequence covered by peptides in MS/MS analysis; MASCOT score = >40 indicates identification or extensive homology (p<0.05).

doi:10.1371/journal.pone.0027193.t005
to kidney bean. ELISA was carried out on solid phase coated kidney bean (2 ng/100 μl/well) and preincubated sera. doi:10.1371/journal.pone.0027193.g004

Mitra et al. [37], chickpea (15.3%) was a potent sensitizer in the present study also (18.0%). sIgE was also found elevated in hen’s egg (35.2%), black gram (22.0%), and pigeon pea (18.0%). Patients showed signs of recovery from the symptoms once they avoided the suspected food, this further confirmed sensitization in them.

Earlier, the biopropensity of few allergenic motifs have been determined by histamine release assay [38,39]. In utility has also been depicted in the diagnosis of hen’s egg, cow’s milk, and wheat allergy with an efficiency of more than 70% [40]. In the present study, kidney bean induced significant histamine release from basophils sensitized with kidney bean positive patients’ sera which correlated significantly (p<0.05) with SPT and sIgE. This suggests the importance of the combination of SPT, sIgE and histamine release in the diagnosis of food allergy, as these three parameters are found to be significantly correlated with each other.

Figure 4. IgE ELISA inhibition of kidney bean extract with self protein as Inhibitor. Kidney bean positive patients’ pooled sera (0.10 v/v) was preincubated with 5, 10, 50, 100, 1000, 10000 ng of three batches of kidney bean extract as Inhibitors (Kb1, Kb2, Kb3), bromelain and CL. (Curvularia lunata) were also used as inhibitor for IgE inhibition to kidney bean. ELISA was carried out on solid phase coated kidney bean (2 ng/100 μl/well) and preincubated sera. doi:10.1371/journal.pone.0027193.g004

IgE ELISA inhibition of kidney bean extract with self and heterologous legume extracts. For determination of cross reactive proteins between kidney bean and different legume extracts, 7 separate serum pools were prepared with sera of patients’ skin test positive to kidney bean but negative to that of legume extract to be tested. Kidney bean positive patients’ pooled sera (0.10 v/v) was preincubated with 5, 10, 30, 100, 1000, 10000 ng of legume extracts namely black gram, chickpea, kidney bean, lentil, pea, soybean and pigeon pea as inhibitors. ELISA was carried out on solid phase coated kidney bean (2 ng/100 μl/well) and preincubated sera. doi:10.1371/journal.pone.0027193.g005

Figure 6. Immunoblot inhibition of kidney bean extract with self and other legume extracts. Kidney bean positive pooled patients’ sera was preincubated with 500 ng of extracts namely kidney bean (lane 1), peanut (lane 2), black gram (lane 3), soybean (lane 4), lentil (lane 5), pigeon pea (lane 6) and pea (lane 7). Bromelain (lane 8) and Convulvulus lunata (lane 9) were also used as inhibitor to assess non specific binding. Immunoblot using kidney bean positive pooled patients’ sera without inhibitor was used as positive control (lane 10) and normal human sera (lane 11) as negative control.

doi:10.1371/journal.pone.0027193.g006

Immunoblotting with hypersensitive patients’ sera has been used successfully to identify IgE binding components of allergen extracts [14,17,37]. Pausani et al. [14] showed 16 IgE-binding proteins in lentil, of which 34 and 38 kDa proteins were important allergens while Ibanez et al. [17] showed six allergenic proteins between 69 and 18 kDa recognized by more than 50.0% of patients, and a 53 kDa protein by 92.0% of the patients. Paul et al. [13] reported five major allergens between 70 and 20 kDa in chickpea. Kumari et al. [14] identified 8 major IgE binding components of 78-16 kDa in black gram. In the present study, immunoblotting demonstrated eight major allergens of 58, 50, 45, 42, 40, 37, 34, 18 kDa in raw kidney bean extract detected by 45.0-95.0% of patients’ sera. Boiling resulted in the loss of high molecular weights (>50 kDa) IgE binding proteins.

The 8 major allergens of kidney bean identified by immunoblotting were subjected to MALD-MS/MS analysis. Of these, 4 showed significant matches to known proteins in database. Two proteins were identified as lectin and alpha-amylase inhibitor, these are known to provide defence against insects, [41] one as phasolin, the main reserve globulin in kidney bean and the fourth belongs to late endospermogenesis-abundant (LEA) proteins with a role in protecting other proteins from aggregation due to desiccation or osmotic stresses associated with low temperature [42]. Rouge et al. [19] also identified phasolin and PHA as possible allergens in kidney bean. Both phasolin (>150 kDa) and PHA (120 kDa) consist of oligomeric proteins resistant to heat denaturation and digestive proteolysis, exhibiting an extended surface susceptible to display IgE-binding epitopes that probably account for their allergenic propensity. Phasolin and PHA are closely related to the Ara h 1 violin and the Ara h 1 allergin (PNA) allergens from pea. In addition to the cupin allergens, lectins appear to be potentially allergenic proteins of edible legumes [19].

Cross reactivity has been reported previously among some legume allergens by ELISA inhibition [14,33]. In the present study, kidney bean required 67.3 μg of homologous protein for 50.0% inhibition of IgE binding in ELISA whereas black gram and peanut produced same inhibition with 85 μg of protein showing extensive cross reactivity. Other legume extracts such as

Figure 5. IgE ELISA inhibition of kidney bean extract with self protein as Inhibitor. Kidney bean positive patients’ pooled sera (0.10 v/v) was preincubated with 5, 10, 50, 100, 1000, 10000 ng of three batches of kidney bean extract as Inhibitors (Kb1, Kb2, Kb3), bromelain and CL. (Curvularia lunata) were also used as inhibitor for IgE inhibition to kidney bean. ELISA was carried out on solid phase coated kidney bean (2 ng/100 μl/well) and preincubated sera. doi:10.1371/journal.pone.0027193.g004

Immunoblotting demonstrated eight major allergens of 58, 50, 45, 42, 40, 37, 34, 18 kDa in raw kidney bean extract detected by 45.0-95.0% of patients’ sera. Boiling resulted in the loss of high molecular weights (>50 kDa) IgE binding proteins.

The 8 major allergens of kidney bean identified by immunoblotting were subjected to MALD-MS/MS analysis. Of these, 4 showed significant matches to known proteins in database. Two proteins were identified as lectin and alpha-amylase inhibitor, these are known to provide defence against insects, [41] one as phasolin, the main reserve globulin in kidney bean and the fourth belongs to late endospermogenesis-abundant (LEA) proteins with a role in protecting other proteins from aggregation due to desiccation or osmotic stresses associated with low temperature [42]. Rouge et al. [19] also identified phasolin and PHA as possible allergens in kidney bean. Both phasolin (>150 kDa) and PHA (120 kDa) consist of oligomeric proteins resistant to heat denaturation and digestive proteolysis, exhibiting an extended surface susceptible to display IgE-binding epitopes that probably account for their allergenic propensity. Phasolin and PHA are closely related to the Ara h 1 violin and the Ara h 1 allergin (PNA) allergens from pea. In addition to the cupin allergens, lectins appear to be potentially allergenic proteins of edible legumes [19].

Cross reactivity has been reported previously among some legume allergens by ELISA inhibition [14,33]. In the present study, kidney bean required 67.3 μg of homologous protein for 50.0% inhibition of IgE binding in ELISA whereas black gram and peanut produced same inhibition with 85 μg of protein showing extensive cross reactivity. Other legume extracts such as
Legume Sensitization and Allergens of Kidney Bean

pigeons presented 30.0% inhibition with 1000 mg protein whereas both chickpea and lentil required 7500 mg of protein for the same. Further, IgE binding protein components of kidney bean were completely inhibited when pooled patients’ sera was preabsorbed with 500 mg of self proteins in immunoblot analysis. Pigeon, pigeon pea and black gram could induce protein bands of 120, 95, 70, 56, 27, 24, 18, and 16 KDa in kidney bean suggesting presence of shared allergens.

In conclusion, kidney beans as well as chickpea, peanut, cowpea and lentil are recognized as important sensitizers by SPT and ELISA. In addition, we have identified 8 major IgE binding protein components from kidney bean and demonstrated its cross-reactivity with peanut, black gram and pigeon pea. Although DBPCFC test is considered to be the gold standard in the diagnosis of food allergy but it presents practical as well as ethical problems and suffers from several pitfalls. Furthermore, DBPCFC is difficult to perform in normal outpatient clinics, therefore, this protocol is rarely performed outside the academic context [30].

We demonstrated three parameters SPT, dIL and basophil histamine release to be significantly correlated with each other and therefore important tool in the diagnosis of food sensitization.

Acknowledgments

Authors are thankful to Dr. P.C. Kathuria, Delhi and Dr. Richard E. Goodman, Nebraska for their helpful suggestions.

Author Contributions

Conceived and designed the experiments: AS BS. Performed the experiments: BK RA. Analyzed the data: RA. Contributed reagents/materials/analysis tools: BK. Wrote the paper: RA BK BS.

References

Effect of thermal processing and γ-irradiation on allergenicity of legume proteins

Ramkrashan Kasera\textsuperscript{a,b}, Anand B. Singh\textsuperscript{c}, Raj Kumar\textsuperscript{d}, Shakuntala Lavasa\textsuperscript{e}, Komarla Nagendra Prasad\textsuperscript{f}, Naveen Arora\textsuperscript{g,\footnote{Corresponding author at: Allergy and Immunology Section, CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India. E-mail address: naveen@igib.res.in (N. Arora).}}

\textsuperscript{a} Allergy and Immunology Section, CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India
\textsuperscript{b} University of Pune, Ganeshkhind, Pune 411007, India
\textsuperscript{c} Institute of Nuclear Medicine and Allied Sciences, Delhi 110007, India
\textsuperscript{d} ILgammagen Research Center, Chandigarh 160015, India
\textsuperscript{e} Bangalore Allergy Centre, Bangalore 560027, India
\textsuperscript{f} University of Pune, Ganeshkhind, Pune 411007, India
\textsuperscript{g} Corresponding author at: Allergy and Immunology Section, CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India. E-mail address: naveen@igib.res.in (N. Arora).

**Abstract**

Legumes are implicated in IgE-mediated food allergy in different countries. The present study aimed to investigate the effect of different processing methods on allergenicity of legume proteins. The extracts were processed by boiling, γ-irradiation or by combination of both. The changes in soluble protein content, specific IgE binding and allergenic potential of legume proteins were assessed. Thermal processing resulted in a 5- to 4-fold reduction in soluble protein. Specific IgE binding was reduced 74 ± 5.2, 83 ± 11.8% and 62 ± 7.2% in the soluble protein of kidney bean, black gram and peanut, respectively, after boiling (p < 0.001) whereas there was 34 ± 5.2, 74 ± 15.6% and 44 ± 11.1% IgE binding reduction in the insoluble protein fraction of respective legumes. Boiling followed by γ-irradiation reduced IgE binding significantly (p < 0.05). Biopotency of soluble protein of kidney bean, black gram and peanut was reduced 7-, 3- and 26-folds (p < 0.001), respectively, and that of insoluble protein decreased 6-, 4- and 8-folds (p < 0.001), respectively, after boiling. Combination treatment was effective in reducing the potency of both soluble and insoluble protein significantly as compared to boiling alone (p < 0.001). However, γ-irradiation alone did not bring any change in allergenicity. In conclusion, boiling followed by γ-irradiation is effective in attenuating allergenicity of legume proteins.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Legumes are important dietary components and are rich source of proteins used in bakery, confectionery and snacks throughout the world. Despite economic and health importance, legumes are implicated in food allergy in sensitive individuals (Zacharisen and Kurup, 1998; Bock et al., 2001; Sicherer and Sampson, 2009; Kumar et al., 2006; Kasera et al., 2011). Currently, there is no definitive treatment available for food allergy. Elimination of food allergen from diet is the best way to avoid adverse reactions, but that is not always feasible. Hence, the approach that could reduce the allergenicity without altering the nutritional value would be beneficial to allergic individuals.

The foods are subjected to a variety of processing conditions, which can cause alterations in epitopes and affect allergenic properties of proteins (Sathe et al., 2005). Thermal processing includes moist or dry heating of foods that leads to reduced allergenicity e.g. in pollen-related fresh fruits and vegetable food allergens or no significant effect e.g. in heat stable shrimp allergen (Sathe et al., 2005). Among non-thermal processing methods, γ-irradiation has been used to control foodborne pathogens, reduce microbial load and insect infestation, inhibit the germination of root crops and extend the shelf life of perishable products. Irradiation has been reported to reduce the antigenicity of ovalbumin, bovine serum albumin and milk protein as well as that of shrimp tropomyosin (Kume and Matsuda (1995); Lee et al., 2001; Byun et al., 2002).

Previously, a heat treated 35 kDa major shrimp allergen was reported to retain its activity even after peptide fragmentation (Hoffman et al., 1981; Hefle, 1996; Latier et al., 2001). Later, a combination of γ-irradiation and heat decreased its allergenicity (Zhenxing et al., 2007). Contrary to this, almond, cashew nut and walnut proteins when exposed to γ-irradiation (1–25 kGy) alone or followed by various thermal treatments maintained their allergenic potential (Su et al., 2004). The effect of γ-irradiation on the nutritional quality of peanut, kidney bean, velvet bean seeds, wheat, many cereals and vegetables were studied and no significant effect
was observed on their nutritional quality (Aziz et al., 2006; Fan and Sokurai, 2008; Budt et al., 2008; Neur et al., 2009). The present study was aimed to determine the effect of thermal processing, γ-irradiation or both on stability of allergenic proteins of kidney bean, black gram and peanut.

2. Materials and methods

2.1. Processing of food materials

2.1.1. Thermal processing

Whole raw legume seeds of kidney bean, black gram and peanut were subjected to boiling at 121 °C for 15 psi for 15 min. The boiled seeds were freeze dried and powdered using mortar and pestle.

2.1.2. γ-Irradiation

Whole raw legume seeds of kidney bean, black gram and peanut were powdered and subjected to γ-irradiation (5, 10, 15, 20 and 25 kGy) at Institute of Nuclear Medicine and Allied Sciences (INMAS), Delhi. The source of γ-irradiation used in the present study was Cobalt-60.

2.1.3. Combination processing using thermal and γ-irradiation

Whole raw legume seeds of kidney bean, black gram and peanut were subjected to boiling at 121 °C (15 psi) for 15 mins and then subjected to γ-irradiation (25 kGy).

2.1.4. Preparation of extracts

Raw extracts of kidney bean, black gram and peanut were subjected to boiling at 121 °C (15 psi) for 15 mins and then subjected to γ-irradiation (25 kGy).

2.2. Processing of raw extracts

2.2.1. Thermal processing

Raw extracts of kidney bean, black gram and peanut were subjected to boiling at 121 °C (15 psi) for 15 mins and then subjected to γ-irradiation (25 kGy).

2.2.2. Combination processing using thermal and γ-irradiation

Raw legume seed extracts were boiled (121 °C for 15 min) and then subjected to γ-irradiation (25 kGy). Processed raw extracts were centrifuged and precipitated proteins were separated for its allergenicity testing as described above. Lyophilized raw extract suspended in urea-thiourea buffer was used as control in allergenicity assessment of precipitated part of protein.

2.2.3. Study subjects

The study included allergic rhinitis and asthma patients (mean age 30.7 ± 13.9) with confirmed sensitization history to the three legumes, kidney bean, black gram and peanut. The diagnosis of asthma and chronic bronchitis was ascertained following American Thoracic Society Guidelines (1991) and Allergic Rhinitis and its Impact on Asthma guidelines (Bousquet et al., 2001), respectively. The study protocol was approved by Human Ethics Committee of Institute of Genomics and Integrative Biology, Delhi. Informed written consents were obtained from all the patients and controls for participation in the study. SPT were performed with raw legume seed extracts and processed legume seed extracts. Processed proteins were used for allergenicity testing. This precipitated protein was resuspended in urea-thiourea buffer (0.05 M urea, 0.01 M thiourea) and stored in small aliquots at -70 °C. Protein content of extracts was determined by Lowry's method with a slight modification by precipitation of proteins using phosphotungstic acid (Singh et al., 1992).

2.2.4. Extraction of specific IgE

Levels of specific IgE by ELISA (enzyme linked immuno-sorbent assay) were determined by using in-house method as described earlier (Khare et al., 2011). Briefly, raw or processed legume proteins extracts was coated in carbonate buffer (100 mM, pH 9.0) and blocked with 1% bovine serum albumin (BSA) for 2 h at 37 °C. The plate was washed and blocked with 1:10 v/v dilution of legume positive patients' sera. The plate was washed and incubated with anti-human IgE (Bethyl laboratories; H-2000 v/v). The specific IgE was detected by adding 1:50,000 dilution of alkaline phosphatase conjugated anti-human IgE antibody and the absorbance was read at 492 nm. IgE binding in ELISA was also elucidated using various processed antigens.

2.2.5. IgE inhibition assay

IgE inhibition assay was performed by ELISA inhibition (competitive ELISA) using hypersensitive pooled patient's sera. Legume seed extract (1 mg/ml) per well was coated in carbonate buffer overnight at 4 °C in a microtiter plate. The patient's pooled serum (1:10 v/v) for respective antigens was preincubated with 10, 50, 100, 500, 1000 and 10,000 ng of self proteins at 4 °C overnight and the mixture was then added to the microtiter plate.
any significant change in the solubility of protein in dry seed powders (0.95 ± 0.06) and peanut (3.70 ± 0.12) as compared to raw kidney bean protein required for 50% inhibition of IgE binding was calculated using the formula (mean ± SD) of all the antigens were significantly reduced to 61-83% (mean 74 ± 6.5%) (p < 0.001) and peanut (43-68%, mean 52 ± 8.4%) (p = 0.039), black gram (56-96%, mean 87 ± 11.6%) and peanut (65-87%, mean 73 ± 7.5%) as compared to their respective raw antigens (p < 0.001) (Table 1, Fig. 3). A significant decrease in the IgE binding was observed in boiled + γ-irradiated kidney bean soluble protein when compared with boiled kidney bean soluble protein (p = 0.011).

The IgE binding to boiled proteins (insoluble) of kidney bean was reduced to 27-46% (mean 34 ± 5.2%) (p > 0.05), that of black gram was reduced to 41-95% (mean 74 ± 15.6%) (p < 0.001) and in peanut it was reduced to 28-63% (mean 44 ± 11.1%) (p < 0.001), among the 10 individuals as compared to IgE binding to their respective raw antigens (Table 1, Fig. 3). The IgE binding was reduced further when combination of boiling and γ-irradiation treatment was used for processing as compared to single processing by boiling in kidney bean (85-93%, mean 89 ± 3.3%), black gram (61-99%, mean 87 ± 11.6%) and peanut (65-87%, mean 73 ± 7.5%) as compared to their respective raw antigens (p < 0.001) (Table 1, Fig. 3). A significant decrease in the IgE binding was observed in boiled + γ-irradiated kidney bean soluble protein when compared with boiled kidney bean soluble protein (p = 0.011).

3. Results
3.1. Soluble protein concentration of legumes

Boiling at 121°C for 15 min led to a significant 3- to 4-fold reduction (p < 0.05) in the soluble protein content (mg/ml) of all three legumes namely kidney bean (1.30 ± 0.08), black gram (2.46 ± 0.06) and peanut (3.70 ± 0.12) as compared to raw kidney bean (4.90 ± 0.20), black gram (4.30 ± 0.32) and peanut (11.3 ± 0.28). Processing by γ-irradiation alone did not produce any significant change in the solubility of protein in dry seed powders and even in the raw extracts of the three legumes as the protein concentration in the antigenic extract was same in irradiated and unirradiated samples. Boiling + γ-irradiation in combination reduced the soluble protein content of kidney bean (1.25 ± 0.10), black gram (0.87 ± 0.06) and peanut (3.55 ± 0.11) as compared to their respective raw extracts. There was no significant difference in the protein concentration among boiled and boiled + γ-irradiated legume seed extracts (p > 0.05) (Fig. 2).

3.2. Specific IgE binding

ELISA was carried out to assess the allergenicity of raw and processed legume extracts, using individual patient's sera with allergy to respective legumes namely kidney bean, black gram and peanut. The IgE binding to thermally processed protein (soluble) of kidney bean was significantly reduced to 61-83% (mean 74 ± 6.5%) (p < 0.001), that of black gram was reduced to 60-68% (mean 83 ± 11.8%) (p < 0.001) and its peanut was reduced to 54-78% (mean 62 ± 7.2%) (p < 0.001), among the 10 individuals as compared to IgE binding to their respective raw antigens (Table 1, Fig. 3). The IgE binding was reduced further when combination of boiling and γ-irradiation treatment was used for processing as compared to single processing by boiling in kidney bean (85-93%, mean 89 ± 3.3%), black gram (61-99%, mean 87 ± 11.6%) and peanut (65-87%, mean 73 ± 7.5%) as compared to their respective raw antigens (p < 0.001) (Table 1, Fig. 3). A significant decrease in the IgE binding was observed in boiled + γ-irradiated kidney bean soluble protein when compared with boiled kidney bean soluble protein (p = 0.011).

The IgE binding to boiled proteins (insoluble) of kidney bean was reduced to 27-46% (mean 34 ± 5.2%) (p > 0.05), that of black gram was reduced to 41-95% (mean 74 ± 15.6%) (p < 0.001) and in peanut it was reduced to 28-63% (mean 44 ± 11.1%) (p < 0.001), among the 10 individuals as compared to IgE binding to their respective raw antigens (Table 1, Fig. 3). The IgE binding to boiled + γ-irradiated protein (precipitate) was reduced further in kidney bean (40%, mean 62 ± 7.2%) (p = 0.001), that of black gram was reduced to 60-90% (mean 87 ± 12.3%) (p = 0.039), black gram (56-96%, mean 87 ± 12.3%) (p < 0.001) and peanut (43-68%, mean 52 ± 8.4%) (p < 0.001) as compared to their respective raw antigens (Table 1, Fig. 3).

No change in the IgE reactivity of γ-irradiated kidney bean, black gram and peanut protein extracts was observed as compared to raw extracts (data not shown). These results suggest that combination treatment i.e. boiling followed by γ-irradiation is significantly better in reducing the allergenicity.
Fig. 3. Specific IgE against raw, boiled soluble (BS), boiled precipitate (BP), boiled soluble + γ-irradiated (BS + γ) and boiled precipitate + γ-irradiated (BP + γ) extracts of kidney bean (A), black gram (B) and peanut (C). A significant difference in specific IgE binding was observed among raw vs BS, raw vs BS + γ, raw vs BP + γ.

3.3. Reduction in allergenic potency of legume proteins after heat treatment and γ-irradiation

Boiling significantly decreased the allergenic potential of kidney bean (7-folds), black gram (3-folds) and peanut (26-folds) (p < 0.001) in soluble part of protein when IC₅₀ was compared to their respective unprocessed seed extracts (Table 2, Fig. 4A-C).

Combined processing by heat + γ-irradiation decreased the allergenic potential of kidney bean (10-folds), black gram (3-folds) and peanut (47-folds) in soluble part of protein further as compared to processing by single method (p < 0.001) (Table 2, Fig 4A-C).

Boiling also brought a reduction in allergenic potential of precipitated protein of kidney bean (6-folds), black gram (4-folds) and peanut (7-folds) in precipitated protein when IC₅₀ was compared to their respective unprocessed seed extracts (Table 2, Fig. 4A-C).

Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Kidney bean protein (ng ± SD)</th>
<th>Black gram protein (ng ± SD)</th>
<th>Peanut protein (ng ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>100 ± 0.50</td>
<td>520 ± 34.77</td>
<td>34 ± 6.00</td>
</tr>
<tr>
<td>Boiled 121 °C + γ</td>
<td>98 ± 0.30</td>
<td>520 ± 13.84</td>
<td>32 ± 5.01</td>
</tr>
<tr>
<td>Boiled 121 °C + γ-25KCy</td>
<td>73 ± 24.82</td>
<td>650 ± 33.40</td>
<td>80 ± 13.16</td>
</tr>
</tbody>
</table>

IC₅₀ of soluble protein of processed and raw legume extracts by ELISA inhibition.
and various processed black gram extracts as inhibitors. EUSA was carried out on separately with 5, 10, 50, 100, 1000, 10,000 ng of their respective raw and various solid phase coated with raw black gram extract (2 |ig/100 nl/well) and preabsorbed preincubated separately with 5, 10, 50, 100, 1000, 10,000 ng of their respective raw processing as inhibitor. Black gram positive patients’ pooled sera (1:10 v/v) were sorbed sera. (B) EUSA inhibition with raw and precipitated black gram protein after 5A-C).

and various processed kidney bean extracts as inhibitors. EUSA was carried out on solid phase coated with raw kidney bean extract (2 |ig/lOO ^l/well) and preab-

and peanut (8-folds) which was also significant (p = 0.001) [Table 3, Fig 5A-C]. Combined processing further brought reduction in allergenic potential of kidney bean (13-folds), black gram (11-folds) and peanut (23-folds) in insoluble (precipitate) protein fractions than the single processing by boiling (p = 0.001) [Table 3, Fig 5A-C].

IC50 of precipitated protein of processed and raw legume extracts by EUSA inhibition.

![Table 3](file)

Boiled + Y-irradiation ppt Boiled ppt Raw Boiled + irradiation ppt Boiled ppt — Raw — Boiled + irradiation ppt Boiled ppt

Discussion

The plant and animal food allergens belong primarily to a few protein families suggesting that certain conserved structure play a key role in determining allergenic properties. In addition, the level of exposure and the physicochemical properties of an allergen contribute tremendously to its allergenic potential (Cochrane et al., 2009). Overall the allergenicity of a food protein is determined by its membership in a certain protein family like storage or defense related proteins, its abundance and stability to processing and digestion (Breiteneder and Radauer, 2004). A majority of the common food allergens are not easily altered by heat treatment, pH change or by proteolytic digestion. Hence, they are more likely to be presented to immune system of the gut as well conserved three dimensional protein structures that are recognized as harmful foreign proteins by immune defenses (Cochrane et al., 2009). In the present study, effect of processing by boiling and/or γ-irradiation on stability of kidney bean, black gram and peanut allergens was investigated.

The extent of loss of protein solubility depends on the type, severity and duration of processing (Zhengxing et al., 2007). In the present study, boiling reduced the soluble protein content 3- to 4-fold in three selected legumes. Boiling significantly reduced specific IgE binding to kidney bean, black gram and peanut in ELISA as compared to their respective raw extracts using individual patient sera. Also a 3- to 26-fold increase in the IC50 value has been observed after thermal processing of legumes as compared to the raw extracts. No difference in the soluble protein concentration, specific IgE binding and IC50 was observed by 7-irradiation at any treated dose as compared to dry legume seeds. These results are in agreement with the previous study of Su et al. (2004) who also reported similar findings based on these three parameters after irradiation of almond, cashew nut and walnut. On the other hand Kume and Matsuda (1995) demonstrated that 8 kGy irradiation was an effective method for reducing allergenicity of ovalbumin and RSA. Conversely, Leszcynska et al. (2003) reported that irradiated gliadin exhibited an increase in IgE binding as determined by ELISA. However, in the present study, boiling followed by γ-irradiation caused a loss in soluble protein content among the three selected legumes and was almost equal to individual processing by boiling. Also, a reduction in specific IgE binding and increase in IC50 was observed

![Fig. 5](file)

(A) Kidney bean

(B) Black gram

(C) Peanut

Conc. in ng

Conc. in ng

Conc. in ng

% inhibition

% inhibition

% inhibition

No significant change in the allergenic potential (IC50) was observed by γ-irradiation at any treated dose as compared to unprocessed materials (p > 0.05). The results demonstrated significant reduction in allergenic potential of legumes after boiling and or combination of boiling followed by γ-irradiation in both soluble and insoluble protein fractions isolated after processing as compared to unprocessed material or with material processed using γ-irradiation alone.

4. Discussion

The plant and animal food allergens belong primarily to a few protein families suggesting that certain conserved structure play a key role in determining allergenic properties. In addition, the level of exposure and the physicochemical properties of an allergen contribute tremendously to its allergenic potential (Cochrane et al., 2009). Overall the allergenicity of a food protein is determined by its membership in a certain protein family like storage or defense related proteins, its abundance and stability to processing and digestion (Breiteneder and Radauer, 2004). A majority of the common food allergens are not easily altered by heat treatment, pH change or by proteolytic digestion. Hence, they are more likely to be presented to immune system of the gut as well conserved three dimensional protein structures that are recognized as harmful foreign proteins by immune defenses (Cochrane et al., 2009). In the present study, effect of processing by boiling and/or γ-irradiation on stability of kidney bean, black gram and peanut allergens was investigated.

The extent of loss of protein solubility depends on the type, severity and duration of processing (Zhengxing et al., 2007). In the present study, boiling reduced the soluble protein content 3- to 4-fold in three selected legumes. Boiling significantly reduced specific IgE binding to kidney bean, black gram and peanut in ELISA as compared to their respective raw extracts using individual patient sera. Also a 3- to 26-fold increase in the IC50 value has been observed after thermal processing of legumes as compared to the raw extracts. No difference in the soluble protein concentration, specific IgE binding and IC50 was observed by 7-irradiation at any treated dose as compared to dry legume seeds. These results are in agreement with the previous study of Su et al. (2004) who also reported similar findings based on these three parameters after irradiation of almond, cashew nut and walnut. On the other hand Kume and Matsuda (1995) demonstrated that 8 kGy irradiation was an effective method for reducing allergenicity of ovalbumin and RSA. Conversely, Leszcynska et al. (2003) reported that irradiated gliadin exhibited an increase in IgE binding as determined by ELISA. However, in the present study, boiling followed by γ-irradiation caused a loss in soluble protein content among the three selected legumes and was almost equal to individual processing by boiling. Also, a reduction in specific IgE binding and increase in IC50 was observed
as compared to the respective raw legumes or legumes processed individually by boiling. Earlier study of Zheng et al. (2007) demonstrated that 5-30 times increase in IC50 value after heat + γ-irradiation on shrimp allergens. Su et al. (2004) did not find any significant change either in the protein profile or in allergenicity of almond, cashew nut and walnut after combined treatment of boiling and γ-irradiation. This again may be attributed to less content of water present in the nuts irradiated. The difference in the results may also be due to the reason that in the study by Su et al. (2004) irradiation is followed by autoclaving in contrast to the present study where autoclaving is followed by irradiation.

Processing by boiling often cause protein aggregation that may lead to loss (in protein solubility). The insoluble fraction might contain certain allergenic proteins(s) that may be worth investigating since individuals consume whole grain of legume(s). However, there is need to convert insoluble protein into soluble form to assess its allergenicity and for that we separated the precipitated part of the extract and re-suspended it in urea/thiourea buffer. The insoluble protein of black gram and peanut obtained after boiling showed a significant decrease in the specific IgG binding as compared to respective raw legumes but a significant decrease in IgG binding was not observed in case of kidney bean. However, decrease in IgG binding became significant in insoluble kidney bean protein when combination processing method was employed. Also a significant (4- to 8-fold) increase in the IC50 was observed after boiling as compared to raw legumes. Analysis of precipitated protein of kidney bean, black gram and peanut obtained after boiling followed by γ-irradiation showed a significant decrease in the specific IgG binding as compared to respective raw legume extracts. Moreover, when combination processing (boiling + γ-irradiation) was employed a further significant (11- to 23-fold) increase in the IC50 was also observed when compared either to raw or boiled extracts.

Dietary supplements and other packaged foods available in India and many countries contain many ingredients (type and quantity) including additives, many in the form of hidden food additives. Many food ingredients (sugar, preservatives, seasonings, colorants) are added during processing in order to prevent or delay the spoilage of food. Cereal grains, appl. radiat. Isot, 64 (12), 1555–1562. As it becomes some­what easier for companies to purchase cheaper products, the use of additives increases. As it becomes somewhat easier for companies to purchase cheaper products, the use of additives increases. As it becomes somewhat easier for companies to purchase cheaper products, the use of additives increases.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

One of the authors (R. Kauser) is a Senior Research fellow of Indian Council of Medical Research, New Delhi.

References


Isolation and characterization of a 28 kDa major allergen from blackgram (Phaseolus mungo)

Dolly Kumari a,1, Naveen Arora a, Ramkrashan Kasera a, h, Susheela Sridhara a, Raj Kumar b, Bhanu Pratap Singh a, h

a Allergy and Immunology Lab, ICMR-Institute of Geonomics and Integrative Biology, Delhi 110007, India
b Department of Biotechnology, University of Pune, Ganeshkhind, Pune 411007, India

ABSTRACT

Legumes are the major elicitors of IgE-mediated food allergy in many countries of the world. Purified major allergens are prerequisite for component resolved diagnosis of allergy. The present study was aimed to isolate and characterize a major allergenic protein from blackgram (Phaseolus mungo). Respiratory allergy patients with history of blackgram allergy were skin prick tested (SPT) and sera were collected from SPT positive patients. The blackgram extract was fractionated using a combination of anion exchange and hydrophobic interaction chromatography. The purified protein was characterized by indirect ELISA, immunoblot, SDS-PAGE, Western blotting and CD analysis. The purified protein separated at 28 kDa on 12% gel and showed IgE binding with 81% of blackgram hypersensitive patients’ sera on immunoblot indicating it to be a major allergen. The purified protein degraded within 5 min after incubation with SIP. The N-terminus-12 residues sequence of 28 kDa protein remained stable up to 15 min on incubation with SCF. The purified 28 kDa protein is a potent major allergen that may have implication in diagnosis of blackgram allergy.

© 2012 Elsevier GmbH. All rights reserved.
Preparation of extracts

Raw blackgram seeds were crushed, powdered and then defatted in diethyl ether at 4°C. The extraction was carried out in 1:20 (w/v) ammonium bicarbonate buffer (50 mM, pH 8) as reported earlier (Kumari et al. 2006). The extracts were centrifuged at 10,000 x g, supernatant was filtered using a 0.22 μm membrane, and freeze dried in small aliquots. Protein content of extracts was estimated by Lowry's method (Lowry et al. 1951).

Methods

Preparation of extracts

Raw blackgram seeds were crushed, powdered and then defatted in diethyl ether at 4°C. The extraction was carried out in 1:20 (w/v) ammonium bicarbonate buffer (50 mM, pH 8) as reported earlier (Kumari et al. 2006). The extracts were centrifuged at 10,000 x g, supernatant was filtered using a 0.22 μm membrane, and freeze dried in small aliquots. Protein content of extracts was estimated by Lowry's method (Lowry et al. 1951).

Study subjects

Respiratory allergy patients with history of food allergy were skin prick tested (SPT) with black gram (Vigna mungo) at the Out Patients Department, V.P. Chest Institute, Delhi. Sera from patients (n = 22) with marked positive skin reactions and elevated specific IgE to black gram extract were collected for immunological experiments. The present study protocol was approved by the Human Ethics Committee of Institute of Genomics and Integrative Biology, Delhi. Informed written consent was obtained from patients and nonallergic volunteers for participation in the study.

Purification of an allergenic protein from blackgram

The whole seed black gram extract was subjected to an ion exchange chromatography using fast protein liquid chromatography. Lyophilized protein (50 mg) was reconstituted in 20 mM Tris buffer (pH 7.9) and loaded onto 5 ml Q-Sepharose gel column equilibrated with same buffer. After washing unbound protein in equilibration buffer, the bound proteins were eluted with a linear salt gradient of 0-400 mM NaCl. All the fractions of each resolved peak were pooled, dialyzed, lyophilized and run on SDS-PAGE. The fractions were tested for IgE binding on immunoblot using pooled patients' sera. Fractions containing a distinct band were collected, dialyzed against PBS, filtered sterilized and finally lyophilized and reconstituted to protein concentration of 10 mg/ml.

ELISA inhibition

The allergenic potency of purified 28 kDa blackgram protein was determined by ELISA inhibition. Inhibition of IgE binding was assessed with serum pools of blackgram positive patients' preincubated with 6.1, 1.10, 100, 1000 and 10,000 μg of 28 kDa purified protein and raw black gram extract as inhibitors. The mixture was added to the solid phase bound raw black gram extract in ELISA plate. Here blackgram positive patients' pooled sera without inhibitor was taken as a positive control. ELISA inhibition was also carried out using Curvularia lunata extract as an inhibitor. Percent inhibition was calculated as described below:

\[
\text{Percent inhibition} = \frac{OD_{sample} - OD_{inhibitor}}{OD_{positive\ control}} \times 100
\]

SDS-PAGE resolved proteins were stained with Coomassie brilliant blue (CBB) or silver stained.

IgE immunoblot

SDS-PAGE resolved purified protein/blackgram extract was transferred onto nitrocellulose membrane (NCM) as described earlier (Towbin et al. 1979). The unbound sites were blocked with 3% BSA in PBS (0.05%) and incubated with 1:1000 (v/v) anti-IgE antibodies. The strips were washed and incubated with 1:1000 (v/v) anti-human IgG horseradish peroxidase (Sigma). The IgE binding was detected by adding 3,3-diaminobenzidine with hydrogen peroxide in sodium acetate buffer (pH=5.0)

Skin test with blackgram extract and purified protein

Skin prick tests (SPTs) were carried out with blackgram extract (1: 500 w/v) in PBS and purified protein (100 μg/ml) protein in PBS at V.P. Chest Institute, Delhi on respiratory allergy patients with history of food allergy. SPT was also performed with a panel of common inhalant allergens such as pollen, fungi and insects. Cytosorbent (50%) phylonyl buffer was used as a negative control and histamine dihydrochloride (5 mg/ml) as a positive control. The skin tests were graded after 20 min in comparison to the wheal diameter of positive control. Wheal diameter equal to that of positive control or more (>3 mm) were considered as marked positive.

Please cite this article in press as: Kumari, D., et al., Isolation and characterization of a 28 kDa major allergen from blackgram (Vigna mungo). Immunobiology (2012), doi:10.1016/j.imbio.2011.12.001
skin reactions. Blood was collected from patients (n = 9) showing 
marked positive reaction to blackgram extract and purified protein.

Stripped basophil histamine release assay

The leucocyte suspension was prepared and basophils stripped off 
their IgE as described by Kleine Budde et al. (2001). The cells 
were then passively sensitized by incubation (37°C, 90 min) with 
150 µl of hypersensitive individual patient’s serum (n = 9) in 4 mM 
EDTA and 10 ng/ml heparin. As a control, basophils were also sen-
sitized with serum pool from healthy individuals (n = 3).

After sensitization, cells were passively sensitized with purified protein 
or blackgram extract (10 ng/ml) diluted in HEPES buffer. Reaction was 
stopped by the addition of 750 µl of ice cold 0.9% NaCl (w/v). His-
tamine released was determined as described earlier (Kleine Budde 
et al. 2001). A histamine release of more than 10% was considered 
positive.

Lymphocyte proliferation and cytokine analysis on allergen challenge

Peripheral blood mononuclear cells (PBMCs) were isolated 
from heparinized blood of blackgram hypersensitive patients' 
(n = 9) by Ficoll Hypaque centrifugation (Sigma Chemical Co.). 
Cells were washed 3 times with PBS and resuspended in RPMI-1640 supplemented with 23 mM sodium bicarbonate, 25 mM HEPES, 
non-essential amino acids and vitamins, streptomycin (100 U/ml), 
pencillin (100 µg/ml) and 10% fetal calf serum. Cell viability was 
determined by adding 20 µl of trypan blue to 20 µl of cells and the 
visible number of cells were adjusted to 10^6 cells/ml. For lympho-
proliferation assay, 100 µl of cells (10^6 cells/well) were cultured 
in RPMI media and challenged with 10 µg of blackgram extract or 
purified protein. Phytotanaemagglutinin (10 µg) was used as positive 
control and no stimulant was added to negative control. The cul-
ture plates were incubated for 72 h and supernatant collected for 
determination of proliferation on MTT (100 µg/ml). The cells were lysed in acidic isopropanol 
and destaining, the protein band was cut and used for N-terminal 
analyse on allergen challenge.

The digestibility of purified protein and blackgram was exam-
inied in simulated gastric fluid (SGF), as described by Ashwood et al. (1996). SGF was prepared as described in the United States Phar-
macopoea (Singh et al. 2006; Sharma et al. 2011) containing 0.01 M 
NaCl, 0.2 (w/v) percentage of pepsin A (Sigma Chemical Co.), pH 1.2. Briefly, 28 kDa purified protein (880 µg) was treated separately 
with 200 µl of pepsin A in SGF. Digestion was proceeded at 37°C 
with continuous shaking, and an aliquot (20 µl) of this digest was 
periodically withdrawn (at 0.5, 1, 5, 15, 30, 45, and 60 min) for anal-
ysis. These aliquots were quickly mixed with 26 µl of a sample 
buffer (containing 2% p-mercaptoethanol and 4% SDS) for SDS-
PAGE together with 6 µl of Na2C03 solution (200 mM/L). The mixture 
was then boiled for 5 min and stored at -20°C until further 
analyses. As control, each protein sample was treated with SGF that 
did not contain pepsin A and then processed as described above.

The purified protein was also digested in simulated intestinal fluid 
(SIF). SIF was prepared and used in the United States Pharmaco-
poea (Singh et al. 2006) containing 10 mg/ml of pancreatin 
(Sigma Chemical Co.) in 0.05 M HEPES, pH 7.5. Purified protein was 
digested in SIF by incubation at 37°C for 25, 1, 4, 8, 16 and 24 h. The 
ratio of pancreatin to test protein was about 13:1 (w/w). The reac-
tion was immediately stopped by placing the tubes in boiling water 
for 10 min. The end products were visualized by SDS-PAGE 
(Lammeli 1970).

Protease activity

To determine the protease activity, 50 µg purified protein and 
blackgram extract (5 µg/ml in PBS) were incubated overnight at 
37°C in the wells perforated in 0.1% agarose gel containing 50 µg of 
bovine serum albumin (BSA), casein hydrolysate or gelatin individ-
ually. A serine protease (Epil 1) from mold Epicoccum purpurascens 
was used as a positive control and PBS was used as a negative con-
trol. The plates were stained with CBB. The lightly stained area 
around the wells indicates the proteolytic activity of protein.

Periodic Acid Schiff’s (PAS) staining

Periodic Acid Schiff’s (PAS) staining was used for detection of 
carbohydrate in purified protein and blackgram extract. For PAS 
staining, approximately 30 µg of purified protein or blackgram 
extract was run on SDS-PAGE. The gel was incubated in 1% peri-
odate in 3% acetic acid solution for 15 min and stained in Schiff’s 
reagent for 15 min in dark. After staining, the gel was washed with 
5% sodium metabisulfite solution for 5 min followed by distilled 
water to visualize the protein bands.

Effect of periodate oxidation on IgE binding

After electro-transfer of proteins onto NCM strip, it was incu-
bated in dark with 20 mM sodium metaperiodate, overnight at 4°C. 
Periodate was inactivated for 5–10 min by adding ethylene gly-
col. To this, 1 mg/ml of sodium borohydride was added and kept 
on ice overnight at 4°C. The reaction was stopped by adding a drop of 
acetic acid. The strip was washed with water and PBS and protein 
free sites were blocked with 3% defatted milk in PBS. The remaining 
steps of western blot were carried out as described earlier (Towbin 
et al. 1979).

N-terminal amino acid sequencing

The purified protein was electrotransferred onto a polyvinyli-
dene difluoride membrane (PDVF). After staining with 0.1% CBB 
and destaining, the protein band was cut and used for N-terminal 
amino acid sequencing on Protein Sequence Prosize 491 (Perkin 
Elmer). The homology of the sequence obtained was determined with 
known proteins in the data bank using BLAST.

Statistical analysis

Correlation coefficients were determined by means of linear 
regression using the Graph pad programme. P value ≥ 0.05 was 
considered significant.

Results

Purification of 28 kDa protein from blackgram extract

The blackgram extract was fractionated into five major peaks 
on Q-Sepharose gel (Fig. 1a). All fractions of each resolved peak 
were pooled, dialyzed, lyophilized and run on SDS-PAGE. The pro-
tein profile of unbound and eluted protein fractions is presented in 
Fig. 1b. Peak 1 showed a distinct band of 28 kDa and two faint bands 
of 47 and 16 kDa. In order to purify the proteins further, fractions of
peak 1 were loaded onto phenyl Sepharose column. The 28 kDa protein appeared in the unbound fraction as a single band on SDS-PAGE and was recognized by blackgram hypersensitive pooled patients’ sera on immunoblot (Fig. 2a and b). The yield of this 28 kDa protein was 1.12 mg/g of blackgram protein.

Allergenicity assessment of purified protein (28 kDa) by in vitro and in vivo methods

Immunosays

Specific IgE binding of purified protein (28 kDa), and blackgram extract was determined by ELISA using 22 blackgram positive patients’ sera. All 22 sera samples showed elevated IgE levels (ODs 0.39-0.92) against blackgram extract whereas 18/22 of sera showed high IgE titer (ODs 0.29-0.63) against purified protein (Table 1). Specific IgE values >3 times of control (blackgram extract OD 0.130, purified protein OD 0.088) were considered positive cut off for ELISA. There was a significant correlation between skin reactivity of purified protein and blackgram extract (r=0.5626, P=0.0064). IgE binding of 28 kDa protein was further assessed by immunoblotting with individual patients’ sera (n=22) having elevated specific IgE levels to blackgram. Of these (n=22), 18 (81.8%) sera showed IgE binding with purified protein demonstrating it to be a major allergen (Fig. 3a). Four patients’ sera that did not show IgE binding to this protein may be sensitized to other protein fractions of blackgram.

Biopotency of 28 kDa protein

Out of 39 respiratory allergy patients skin prick tested with different food extracts, 14 showed marked positive skin reactions with one or more legumes. Of these (n=14), 9 patients showed marked positive skin reaction to blackgram extract while 7 patients with purified 28 kDa protein. There was a significant correlation between skin reactivity of purified protein and blackgram extract (r=0.72, P=0.0035). Non allergic subjects did not demonstrate positive skin reaction with any of the allergen extract. One patient with positive SPT to purified protein showed negative skin reaction to the blackgram extract may be specifically sensitive to this protein.

Specific IgE levels against blackgram extract were elevated in sera from all 9 skin test positive patients (ODs 0.404-0.918) whereas 7 patients demonstrated raised specific IgE titer (OD 0.311-0.547) with purified protein in ELISA. A significant correlation was observed between the IgE response to purified protein and blackgram extract (r=0.68, P=0.0071).
**ELISA inhibition**

The specificity and the allergenic potency of purified 28 kDa protein were assessed by ELISA inhibition using blackgram hypersensitive pooled patients’ sera. A dose dependent inhibition of IgE binding to solid phase blackgram extract was observed, when serum pool was incubated with increasing concentrations (0.1–10,000 ng) of both crude extract and purified protein, separately (Fig. 3b). Approximately 142 ng of purified protein resulted in 50% inhibition of IgE binding to blackgram extract (solid phase), whereas 50% inhibition of IgE binding to solid phase blackgram was achieved by 14 ng of blackgram extract (self extract) used as inhibitor. The purified protein was able to inhibit 61% of the IgE binding to solid-phase blackgram extract in its highest amount used (10 μg) as inhibitor. Less than 10% inhibition of IgE binding to blackgram extract was observed when pooled sera was preincubated with *Curvularia lunata* extract.

**Table 1**

<table>
<thead>
<tr>
<th>Patients no.</th>
<th>Blackgram extract (OD)</th>
<th>Purified 28 kDa protein (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.41</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>0.39</td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>0.92</td>
<td>0.33</td>
</tr>
<tr>
<td>4</td>
<td>0.52</td>
<td>0.29</td>
</tr>
<tr>
<td>5</td>
<td>0.66</td>
<td>0.52</td>
</tr>
<tr>
<td>6</td>
<td>0.71</td>
<td>0.51</td>
</tr>
<tr>
<td>7</td>
<td>0.69</td>
<td>0.39</td>
</tr>
<tr>
<td>8</td>
<td>0.48</td>
<td>0.36</td>
</tr>
<tr>
<td>9</td>
<td>0.66</td>
<td>0.42</td>
</tr>
<tr>
<td>10</td>
<td>0.39</td>
<td>0.31</td>
</tr>
<tr>
<td>11</td>
<td>0.07</td>
<td>0.33</td>
</tr>
<tr>
<td>12</td>
<td>0.42</td>
<td>0.18</td>
</tr>
<tr>
<td>13</td>
<td>0.30</td>
<td>0.17</td>
</tr>
<tr>
<td>14</td>
<td>0.40</td>
<td>0.13</td>
</tr>
<tr>
<td>15</td>
<td>0.66</td>
<td>0.37</td>
</tr>
<tr>
<td>16</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>17</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>18</td>
<td>0.47</td>
<td>0.52</td>
</tr>
<tr>
<td>19</td>
<td>0.58</td>
<td>0.47</td>
</tr>
<tr>
<td>20</td>
<td>0.63</td>
<td>0.62</td>
</tr>
<tr>
<td>21</td>
<td>0.42</td>
<td>0.38</td>
</tr>
<tr>
<td>22</td>
<td>0.52</td>
<td>0.55</td>
</tr>
<tr>
<td>C1</td>
<td>0.13</td>
<td>0.08</td>
</tr>
<tr>
<td>C2</td>
<td>0.13</td>
<td>0.08</td>
</tr>
<tr>
<td>C3</td>
<td>0.13</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Correlation coefficient r = 0.5626, P = 0.0004

*Please cite this article in press as: Kumari, D., et al., Isolation and characterization of a 28 kDa major allergen from blackgram (Phaseolus mungo), Immunobiology (2012), doi:10.1016/j.imbio.2011.12.011*
Fig. 4. (a) Histamine released from stripped basophils re-sensitized with individual patients’ sera (n = 9) on challenge with blackgram extract (•) and 28 kDa purified protein (D). CI - C3: controls. (b) Lymphoproliferation assay. PBMCs from blackgram hypersensitive patients demonstrated significant proliferation when challenged with 5 μg of blackgram (•) extract or purified 28 kDa protein (D), whereas PBMCs from nonallergic controls (CI - C3) did not show significant proliferation. PBMCs stimulated with 5 μg phytohemagglutinin (A) and without stimulation were taken as positive and negative controls (C4 - C5). (c) IL-4 cytokine levels in the culture supernatant on stimulation with blackgram extract (•) and 28 kDa purified protein (D). CI - C3: controls.

Stripped basophil histamine release

The blackgram hypersensitive patients (n = 9) showed positive histamine release upon challenge with 10 ng each of blackgram extract (31.41–65.98%) or purified protein (14.00–59.62%) (Fig. 4a). The controls (n = 3) with negative skin reactivity to both the purified protein and blackgram extract demonstrated <10% histamine release. Statistically significant correlation was observed between histamine release with purified protein and blackgram extract (r = 0.67, P = 0.048).

Lymphoproliferation assay

Lymphocyte proliferation was investigated in blackgram sensitized patients’ PBMCs by stimulation with 5 μg of purified protein or blackgram extract. Purified 28 kDa protein induced significant...
cellular proliferation (>3 times of control) in 8 patients sensitized to blackgram (Fig. 4b). OD values ranged from 0.088 to 0.577 (mean OD 0.421 ± 0.09) on stimulation with purified protein as compared to 0.323-0.699 with blackgram extract. However, no stimulation (mean OD 0.06 ± 0.01) was observed in non allergic individuals’ PBMCs.

The purified 28 kDa protein also induced significantly higher IL-4 release (mean 285 ± 78 pg/ml; range 121-413 pg/ml) as compared to controls (34-72 pg/ml) in cell culture supernatant. IL-4 released on incubation with blackgram extract or purified protein was comparable (r=0.94, P<0.05) (Fig. 4c).

Periodate treatment

The purified 28 kDa protein appeared to be a glycoprotein by PAS staining (Fig. 6a). On periodate treatment, the 28 kDa protein, showed a slight decrease in IgE binding with blackgram hypersensitive pooled patients’ sera on immunoblot (Fig. 6b).

The purified protein and blackgram extract did not show proteolytic activity on defatted milk (data not shown). Similar results were obtained with BSA, gelatin and casein hydrolysate. Purified protein got cleaved by trypsin into ~22 kDa fragment as visualized on SDS-PAGE (data not shown).

Biochemical characterization

Digestion in SGF and SIF

The digestibility of blackgram and purified 28 kDa protein was assessed by SGF and SIF. The 28 kDa protein was digested with in 15 min treatment in SGF. After 15 min, 14-16 kDa fragments/polypeptides appeared that remained stable for 60 min (Fig. 5a). SDS-PAGE separated proteins (digests) were immunoblotted with hypersensitive pooled patients’ sera. The 28 kDa protein and its fragments (14-16 kDa) showed IgE binding on immunoblot (Fig. 5b). The purified protein on digestion with SIF remained visible for 15 min (Fig. 5c) and showed IgE-reactivity with pooled patients’ sera on immunoblotting (Fig. 5d).

N-terminal sequencing

N-terminal sequence of the 28 kDa purified protein showed 12 residues ‘GREDYDNYLQQ’. In BLAST database, a stretch of residues ‘DDYDNLQ.L’ showed homology with Rho-specific inhibitor of transcription termination (E=-0.42, Identity=87%) and NBS-LRR type disease resistant protein from peanut [Arachis hypogaea] (E=-2, Identity=77%).

Discussion

The knowledge of biochemical and immunologic properties of disease-eliciting proteins is essential for the appropriate diagnosis and therapy of type I allergic disorders. Diagnosis of allergy with...
The 28 kDa protein demonstrated significant histamine release in 9 patients' samples hypersensitive to blackgram/purified protein. Purified 28 kDa protein showed IgE binding with more than 80% of hypersensitive patients' sera to blackgram on immunoblot indicating it to be a major allergen. A dose dependent inhibition of IgE binding to the blackgram extract was observed by purified protein in competitive ELISA. One hundred and forty two ng of the purified protein was required to achieve 50% inhibition as compared to 14 ng of the self-extract. A major peanut allergen Ara h 1 caused 50% inhibition at 5.5 ng as compared to 1.5 ng of the self-extract. Up to 61% inhibition was observed with purified 28 kDa protein while up to 88% inhibition could be achieved by preincubation of sera with blackgram extract (at 10 μg/mL). A major lentil allergen Len c 1 inhibited upto 63% IgE binding to crude lentil extract (Sanchez-Monge et al. 2000). The 28 kDa protein demonstrated significant histamine release in 9 patients' samples hypersensitive to blackgram/purified protein. A previous study had shown that mediator release from basophil significantly correlated with airway reactivity in asthmatic patients highlighting the effector function of basophils in inflammatory diseases and the pathologic significance of mediator release (Gaddy and Busse 1986).

Resistance to proteolytic enzymes and heat is a property associated with many food allergens. These produce stable allergen fragments that resist conditions of the gastrointestinal tract, and have the potential to sensitize the immune system. Astwood et al. (1996) showed that food allergens were either resistant to pepsin in SGF or formed stable fragments, whereas the nonallergens were completely digested within 15 s. Stability of the whole protein or fragments from the allergens tested ranged from 8 to 60 min in SGF. Digestion of Ara h 1 by pepsin showed IgE reactive peptide fragments of 16–29 kDa that were stable till 30 min to 3 h whereas treatment with trypsin and chymotrypsin resulted in IgE reactive fragments ranging from 16 to 59 kDa (Malek et al. 2000). In the present study, treatment of 28 kDa protein, with pepsin resulted in fragments of 16-29 kDa that were stable till 30 min to 3 h whereas treatment with trypsin and chymotrypsin resulted in IgE reactive fragments ranging from 16 to 59 kDa (Malek et al. 2000). In the present study, treatment of 28 kDa protein, with pepsin resulted in stable IgE reactive fragments. The 28 kDa protein however digested within 15 min upon treatment with pancreatin. Pancreatin has been a mixture of amylase, trypsin and lipase with trypsin being the only proteolytic component which is assumed to digest rest of the constituent proteins (amylase and lipase). This explains the
reduction in number of bands in the molecular weight range of 20-37 kDa with time. The 28 kDa protein was detected as a glycoprotein by PAS staining. On peroxidase treatment, there was a decrease in IgE binding with blackgram allergens. The purified protein is a 28 kDa protein (Mr 28 kDa). The purified protein seems a potent allergen and may be implicated in possible strategies for component resolved diagnosis and therapy.

Acknowledgements

One of the authors (Dolly Kumari) received Senior Research Fellowship from Council of Scientific and Industrial Research, New Delhi. Thanks are due to Dept. of Biotechnology, New Delhi for financial support to this study.

References


