Chapter 5: Purification and characterization of 31 kDa kidney bean allergen
Chapter 5  
**Purification and characterization of 31 kDa kidney bean allergen**

**INTRODUCTION**

Legumes such as kidney bean, peanut, chick pea, black gram, pigeon pea, lentil etc are an important source of IgE mediated hypersensitivity (Kumari et al., 2006; Misra et al., 2009; Misra et al., 2010; Kasera et al., 2011, Verma et al., 2012). The clinical manifestations of allergy to legumes range from OAS, urticaria, angioedema, rhinitis and asthma (Pereira et al., 2002). Kidney bean is consumed in Latin America, Africa, Middle East and Mediterranean (FAO, 1993). It contains high protein, abundant fibre and essential minerals like iron, zinc, calcium, and phosphorus (Carvalho et al., 2012). Rouge and coworkers have also reported anaphylaxis to kidney bean (Rouge et al., 2011).

Over the years, many allergens have been purified from diverse sources such as pollen, fungi, insects, mites, etc including foods/legumes. Peanut is the most important food allergen effecting both children and adults. Three major allergens, Ara h 1, 2 and 3 (Burks et al., 1991; Burks et al., 1992; Rabjohn et al., 1999), and four minor allergens, Ara h 4, Ara h 5, Ara h 6 and Ara h 7 (Kleber-Janke et al., 1999), were identified from peanut. With the exception of the profilin Ara h 5, these allergens are seed storage proteins. Ara h 8 is a homologue of Bet v 1 and was identified as a major allergen in birch-pollen-allergic patients with concomitant peanut allergy (Mittag et al., 2004). Peanut oleosin, a family of proteins involved in the formation of oil bodies, was also identified as an important peanut allergen. This protein also had cross-reactivity with soybean (Pons et al., 2002). Ara h 9, a lipid transfer protein was identified as a major allergen in peanut among allergic patients from the Mediterranean area (Lauer et al., 2009). Lentil has been ranked as the fourth most important allergen in Spain, where the frequency of its allergy is high in pediatric population. Three important allergens from lentil (Len c 1, Len c 2 and Len c 3), two from soybean (β-conglycinin and n-conglycinin), Pha v 3 from green bean and Vig r 1 from Vigna radiata were identified, isolated and characterized (Mittag et al., 2005; Blanc et al., 2008; Rigby et al. 2008; Sancho et al., 2010; Zoccatelli et al., 2010; Akkerdaas et al., 2012).

Kidney bean is an important legume consumed in most part of the world and its cross-reactivity with other legumes is established (Kasera et al., 2011). Type I allergy has become not only a major health issue but also an economic problem in developed
countries. So far, specific immunotherapy (SIT) is the only approved curative treatment available, but SIT still harbors drawbacks, such as side effects (Bousquet et al., 1998; Valenta, 2002; Larche et al., 2006) and the use of crude allergen extracts (Ball et al., 1999; Moverare et al., 2002). Aqueous extracts used currently are heterogeneous mixture of proteins and glycoproteins and contain 20-50 proteins that are potential allergens (Schou, 1993). Although immunotherapy with offending allergens is practiced in properly selected cases for management of allergic disorders and has shown long-term beneficial effects (Jutel et al., 1995; Ebner et al., 1997; Durham et al. 1998).

However, the current methods for allergy diagnosis/immunotherapy suffer due to the use of whole mass extracts. The extracts show batch-to-batch variation in their allergen content (Chapman et al., 2000; Sriramarao et al., 1993). Immunotherapy with such extracts may result in additional sensitization to irrelevant components and can hamper in reaching the optimal maintenance dose during treatment. Moreover, not all the components present in extracts are allergenically relevant for diagnosis and therapy. To overcome this, use of purified and well-defined allergen preparations has been recommended (Hamilton et al., 2004). The availability of purified proteins paves the way for component-resolved diagnosis and a custom-made therapy. Development and progress made in the field of purified allergens have allowed for the development of a new concept in allergy diagnosis, molecular diagnosis, which makes it possible to identify potential disease-eliciting molecules. Microarray-based testing performed with a small amount of serum sample has enabled clinicians to determine specific IgE antibodies against multiple recombinants or purified natural allergen components. Purified allergens are required to detect cross-contamination with other allergenic foods and also to understand allergen interaction with other components of the food matrix apart from using them for diagnosis and treatment of food allergies. For example, serological methods are being developed to improve the quality of diagnosis, and to reduce the need for food challenge tests (Sancho et al., 2010). The use of allergen components and the successful interpretation of test results in the clinic require some degree of knowledge about the basis of allergen components and their clinical implications. Allergen components can be classified by protein families based on their function and structure (Sastre, 2010). Pure allergens are indispensable as reference materials for the calibration.
and standardization of methods between different laboratories and operators for risk assessment in the food industry. Therefore, there is a need for well-defined purified food allergens (Sancho et al., 2010). Moreover, purified proteins can also be used to develop antibodies which could provide a valuable tool for sensitive detection of respective allergenic products in food (Liu et al., 2012).

Thus, the present investigation was undertaken to isolate and characterize a major allergenic protein of kidney bean using column chromatography and standard immunobiochemical methods.

MATERIALS AND METHODS

Preparation of extracts: The extraction of whole kidney bean antigen was carried out following the protocol as described earlier in chapter 2. Briefly, seed materials were crushed, defatted and the antigens were extracted in 1:20 (w/v) ammonium bicarbonate buffer (50 mM, pH 8.0) with continuous stirring for 8 hrs at 4° C. The suspension was centrifuged at 12,000 rpm for 30 min at 4° C. The supernatant was dialyzed for overnight. The extracts were again centrifuged and membrane filtered (0.22 µm). Protein was estimated in the extract using modified lowry’s method (Singh et al., 1992).

Purification of kidney bean allergen: Three chromatographic steps were used to obtain purified 31 kDa protein.

Ion exchange chromatography: Kidney bean extract was subjected to anion exchange chromatography using fast protein liquid chromatography. Kidney bean extract (75 mg) was reconstituted in 20 mM Tris buffer (pH 7.9) and loaded onto Q Sepharose column equilibrated with the same buffer. After washing away the unbound protein, the bound proteins were eluted with a linear NaCl gradient (0-1000 mM). Fractions of each resolved peak were pooled, dialyzed, lyophilized and run on SDS-PAGE.

Gel filtration chromatography: Pooled fractions containing allergenic protein were further loaded on Superdex™ 75 column equilibrated with 20 mM phosphate buffer, pH
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7.0. Fifty fractions (0.5 mL) were collected and protein content was determined, concentrated, analyzed by immunoblot with hypersensitive pooled kidney bean patients' sera. Allergenic protein fractions were pooled for further purification.

**Reverse-phase hydrophobic chromatography:** Pooled fractions (after gel filtration chromatography) were loaded on C-18 column and further purified using high pressure liquid chromatography (HPLC). Unbound proteins were washed out with distilled water till the absorbance became zero at 280 nm. The bound proteins were eluted with 50% acetonitrile in water containing 0.1% trifluoroacetic acid. The eluted fraction was analyzed by SDS-PAGE and immunoblotting using kidney bean hypersensitive pooled patients' sera.

**Study subjects:** Allergic rhinitis and asthma patients with confirmed history of legume allergy as described in chapter 2 were included.

**Skin prick test:** SPT were performed with kidney bean extract (1:10 w/v) and purified protein in 50% glycerinated solution as described in chapter 2.

**Estimation of Specific IgE:** Levels of specific IgE in SPT positive patients' sera was determined by ELISA by following the protocol described earlier in chapter 2. Briefly, kidney bean extract or the purified protein was coated in carbonate buffer in a microtitre plate. The nonspecific sites were blocked and incubated with 1:10 v/v diluted test sera, overnight at 4° C. The plate was washed and incubated with antihuman IgE-horse radish peroxidase (1:1000 v/v) for 4 h at 37° C. The reaction was stopped by adding 2.5 M H2SO4 and the absorbance was read at 492 nm. A specific IgE value =3 time of control was considered as positive.

**ELISA inhibition assay:** The allergenic potency of crude kidney bean extract and purified protein was determined by ELISA inhibition (competitive ELISA) using hypersensitive pooled patients' sera as described earlier in chapter 2. Briefly, Kidney bean extract (1 µg/100 µl per well) or purified protein was coated in carbonate buffer overnight at 4° C in a microtitre plate. The kidney bean patients' pooled serum (1:10 v/v) for respective antigen was preincubated with 10, 50, 100, 1000 and 10000 ng of self protein at 4° C overnight and the mixture was then added to the microtitre plate coated
with same extract. Pool of normal human sera was used as control. The protein required for 50% inhibition of IgE binding was calculated using the formula given below

\[
\text{OD of sample with inhibitor} - \text{OD of sample without inhibitor}
\]

Competitive ELISA was also performed to determine cross-reactivity of purified protein with other legumes. Here, pooled patients’ sera was inhibited with different legume extracts (peanut, black gram and pigeon pea) and ELISA was performed with preinhibited sera and purified protein on solid phase.

**SDS-PAGE and immunoblotting:** The 31 kDa protein (2.5 μg) was resolved on 12% reducing gel and silver stained for visualization.

For immunoblot, the resolved proteins were transferred on to nitrocellulose membrane as described by earlier in chapter 2. Briefly, the unbound sites were blocked by 3% bovine serum albumin for 3 h at 37° C. The NCM strips were washed and incubated with 1:10 v/v kidney bean-hypersensitive patients’ sera (preinhibited with bromelain) at 4° C. Healthy serum pool was taken as control. The strips were washed with PBST and incubated with 1:1000 diluted antihuman IgE-peroxidase. The IgE binding was detected by diaminobenzidine with hydrogen peroxide in sodium acetate buffer (pH 5.0).

**Stripped Basophil histamine release assay:** Histamine release assay was performed in 15 kidney bean sensitive individuals having significantly high specific IgE values following the protocol described earlier in chapter 2. In brief peripheral blood was drawn from nonallergic donors and basophils were separated. Bound IgE was stripped off using lactic acid buffer. Subsequently, the cells were resensitized with serum IgE of individual patients (n=15) and controls (n=5). The histamine release assay was standardized using a graded amount of protein (1 ng-1 mg), and the protein concentration inducing optimal histamine release (10 ng for crude extract and 5 ng for purified protein) was selected for the assay. After passive sensitization, cells were stimulated with either kidney bean (10 ng) or with purified protein (5 ng) for 1h. The histamine content was determined by the fluorometric method, using o-phthalaldehyde. Spontaneous histamine release was measured in the supernatant of unstimulated cells. The total histamine content was determined by lysis of cells with 3% perchloric acid. The allergen-induced histamine
release was calculated as a percent of the total histamine content after correcting for spontaneous release. A histamine release of more than 10% was considered positive.

Two dimensional gel electrophoresis: It was performed to study the purity of protein. The protein samples were precipitated using a 2DE clean up kit (catalogue no. 80-6484-51, GE Healthcare Biosciences Corporation, Piscataway, NJ), as per instructions from the supplier. The clean proteins were solubilized in rehydration buffer (RB) containing 7.0 M urea, 2.0 M thiourea, 20 mM dithiothreitol (DTT), 0.5% bioampholytes, and 2% 3-(3-cholamidopropyl)-dimethylammonio]-l-propanesulfonate. A total of 4 µg of protein in RB (125 µL) was applied to 7 cm nonlinear (pH 4-7) IPG strips (BioRad, CA, USA) and left overnight for rehydration after overlaying with mineral oil (BioRad, CA, USA). Following incubation, the strips were transferred to the focusing tray. After adding mineral oil over the strips, the separation of proteins in the first dimension was performed in an IEF cell (BioRad, CA, USA) using the standard program. After focusing, a two step equilibration was performed in buffers containing SDS. In step-I, the strips containing immobilized proteins were reduced in buffer containing 6.0 M urea, 2% SDS, 30% glycerol, 2% DTT, and 0.375 M Tris-HCl (pH 8.8) for 20 min. Step-II involved alkylation of reduced proteins in the buffer of same composition except DTT, which was replaced by 2.5% iodoacetamide, again for 20 min. After equilibration the strips were placed over 1 mm thick 12.5% vertical acrylamide gel and held in position with molten 0.4% agarose containing bromophenol dye. The second dimension separation was performed in mini Protean III assembly (BioRad, CA, USA) using Tris-glycine-SDS buffer (250 mM glycine, 25 mM Tris, and 0.1% SDS) until the dye front reached near the bottom edge of the gel.

Silver staining: After electrophoresis, the gels were subjected to silver staining following the method described by Blum et al. (1987). Briefly, the gel was incubated in fixative solution containing 50% methanol and 12% acetic acid on dancing shaker for 1.5 h at room temperature. The fixative was removed and saved for use in later step of the experiment. The gel was washed with 50% ethanol and then with 30% ethanol for 30 min each. Subsequent to washing with ethanol, the gel was treated with sodium thiosulphate solution (0.002%) for 60 sec and washed thrice with distilled water for 20 sec each. The
gel was placed in staining solution (12 mM AgNO₃ and 0.028% formaldehyde) for 20 min. After incubation, the gel was washed with distilled water thrice to remove traces of silver stain and then developed. The developing solution was consisted of sodium carbonate (6%), formaldehyde (0.0185%) and sodium thiosulphate (4%). The gel was then placed in fixative solution for 10 min. After that the gel was washed with double distilled water for 10 min and stored in 5% acetic acid solution. The stained gel was scanned at a resolution of 300 dpi using a scanner to acquire the image.

**Mass spectrometric analysis:** The gels were silver stained, spots were excised and digested with trypsin. The solution was injected into Agilent nanoLC-1100 (Agilent, Palo Alto, CA, USA) for analysis as described in chapter 2.

**Periodic Acid Schiff's (PAS) staining:** It was used for detection of carbohydrate in purified protein and kidney bean extract. For PAS staining, approximately 30 µg of purified protein or kidney bean extract was run on SDS-PAGE. The gel was incubated in 1% periodic acid in 3% acetic acid solution for 15 minutes and stained in Schiff's reagent for 15 minutes in dark. The gel was washed with 5% sodium metabisulfite solution for 5 minutes followed by distilled water to visualize the protein bands. (Kumari et al., 2012).

**Periodate oxidation:** The NCM protein strip was incubated in dark with 20 mM sodium metaperiodate, overnight at 4° C. Periodate was inactivated for 5-10 minutes by adding ethylene glycol, 1 mg/ml of sodium borohydride was added, kept overnight at 4° C and reaction was stopped by adding a drop of acetic acid. The strip was washed with water/PBS and protein free sites were blocked with 3% defatted milk in PBS. The remaining steps of Western blot were carried out as described in Chapter 2. (Kumari et al., 2012).

**Hemagglutination Assay (HA):** Hemagglutination activity of purified 31 kDa protein was determined by HA assay. Briefly, PBS (50 µl/well) was added to 96 well microtitre plate. To the first column either 50 µl purified 31 kDa protein (2 mg/ml) or purified PHA (Sigma Aldrich) was added on microtitre plate. PBS was used as a negative control. Now the protein was serially diluted. Finally, 50 µl of 0.5% red blood cell working solution was added to each well and mixed gently. The plate was left undisturbed at room
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Temperature for 1 h at an angle of 45°. Negative results appeared as dots at the corner of plates. Positive results were observed as a uniform reddish color across the well. Protein HA titre is a simple number of the highest dilution factor that produced a positive result. OD of the supernatant was taken at 410 nm without disturbing the pellet.

**Simulated Gastric Fluid (SGF) Digestion:** The digestibility of purified kidney bean protein was examined in the SGF, as described in chapter 3. Briefly, Purified protein (6.8 μg) was treated with 200 μL of prewarmed SGF (US Pharmacopoeia) containing 0.0032 w/v percentage of pepsin A (Sigma Chemical Co, USA). 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 minutes for analysis on SDS-PAGE.

**Statistical analysis:** Values are represented as mean±SD. Correlation analysis was carried out to study the association among purified and crude protein using MS EXCEL, Prism V software (Graph Pad Prism, San Diego, California, USA). Epi Info 3.3.2. was used to calculate the significance of SPT positivity among purified 31 kDa and crude kidney bean extracts. The significance level was considered to be p<0.05.

**RESULTS**

**Purification of 31 kDa kidney bean protein:** The adsorbed proteins on Q sepharose column were eluted using NaCl gradient and fractions were run on SDS-PAGE. Fraction no. 10 to 14 which showed high intensity band at 31 kDa were pooled (Figure 5.1A) and IgE binding was confirmed by immunoblotting using kidney bean hypersensitive pooled patients’ sera. The protein was further purified using gel filtration and the fractions no. 17 to 19 with 31 kDa fractions (Figure 5.1B) were pooled and purified using C18 columns. The major peak of interest, 31 kDa was eluted at a retention time of 4.57 min, appeared as a single band on SDS-PAGE after silver staining and was recognized by kidney bean hypersensitive pooled patients’ sera on immunoblotting (Figure 5.1C).
Allergenicity assessment of purified 31 kDa protein: Specific IgE binding of purified protein (31 kDa), and kidney bean extract was determined by ELISA using 25 kidney bean sensitive patients’ sera (SPT positive). Specific IgE binding of 31 kDa protein, and kidney bean extract showed elevated IgE levels (OD 0.479 to 3.161) against kidney bean extract whereas 22/25 sera showed positive IgE values (OD 0.390 to 1.137) against purified protein (Table 5.1). There was a significant correlation of specific IgE reactivity of purified protein and kidney bean extract (r=0.676, p<0.01).

IgE binding of 31 kDa protein was further assessed by immunoblotting with individual patients’ sera (n=25) having elevated specific IgE levels to kidney bean. Of these, 22 (88%) sera showed IgE binding with purified protein demonstrating it to be a major allergen (Figure 5.2). Three patients (patient no. 5, 9 and 12) did not show IgE binding to 31 kDa protein. This suggested that these patients might be sensitized to other proteins present in kidney bean.

Biopotency of purified protein: Out of 25 kidney bean positive patients, 31 kDa protein was used to perform SPT on 14 subjects. It was also observed that 11/14 patients showed marked positive skin reaction. There was a significant correlation between skin reactivity of purified protein and kidney bean extract (r=0.615, p=0.0147). Specific IgE levels were significantly elevated in sera from all 11 SPT positive patients against both kidney bean and purified protein in ELISA (Table 5.1). Non allergic subjects did not demonstrate positive skin reaction with any of the allergen extract.

Histamine release: Basophils sensitized with kidney bean sensitive individual patients’ sera exhibited elevated histamine release after challenge with kidney bean extract and the purified 31 kDa protein in the range of 28-67% and 17-48% respectively (Figure 5.3A). Basophils sensitized with control sera released less than 5% histamine. Statistically significant correlation was observed between histamine released by kidney bean extract and 31 kDa protein (r= 0.6903, p<0.01) (Figure 5.3B).

PAS staining and periodate treatment: The 31 kDa protein was identified as a glycoprotein after PAS staining (Figure 5.4A). No change in the IgE binding was observed after periodate treatment on immunoblot with patients’ sera (Figure 5.4B).
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ruled out the possibility of any carbohydrate specific IgE binding (non specific) to 31 kDa protein.

**Figure 5.1**: Protein profile of anion exchange eluted fractions containing 31 kDa as a major eluted protein (A) lane 1-5: fractions collected at 10 to 15 min. Protein profile of gel filtration eluted fractions containing 31 kDa as a major eluted protein (B) lane 1-3: fractions collected at 34 to 40 min. Elution profile of purified 31 kDa kidney bean protein after HPLC (C) lane 1: purified protein after silver staining on SDS-PAGE, lane 2: immunoblot of purified protein using kidney bean hypersensitive pooled patients’ sera.

**Figure 5.2**: IgE binding of purified 31 kDa protein with individual patients’ sera (1-25) on immunoblot. C: purified protein probed with control sera.
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Table 5.1: SPT and specific IgE among the 25 patients against raw kidney bean extract and 31 kDa protein.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/ Sex</th>
<th>Histamine Wheal area (mm²)</th>
<th>Kidney bean extract</th>
<th>31 kDa protein</th>
<th>specific IgE (OD) to kidney bean*</th>
<th>specific IgE (OD) to 31 kDa*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51/F</td>
<td>7x7</td>
<td>6x3</td>
<td>4x4</td>
<td>1.822</td>
<td>1.066</td>
</tr>
<tr>
<td>2</td>
<td>34/F</td>
<td>5x5</td>
<td>6x5</td>
<td>n.d.</td>
<td>2.621</td>
<td>0.562</td>
</tr>
<tr>
<td>3</td>
<td>32/M</td>
<td>7x5</td>
<td>5x4</td>
<td>4x3</td>
<td>0.808</td>
<td>0.491</td>
</tr>
<tr>
<td>4</td>
<td>35/M</td>
<td>5x6</td>
<td>6x5</td>
<td>n.d.</td>
<td>2.318</td>
<td>1.034</td>
</tr>
<tr>
<td>5</td>
<td>56/F</td>
<td>7x4</td>
<td>4x3</td>
<td>2x2</td>
<td>0.426</td>
<td>0.071</td>
</tr>
<tr>
<td>6</td>
<td>34/M</td>
<td>7x4</td>
<td>5x5</td>
<td>5x4</td>
<td>1.262</td>
<td>0.909</td>
</tr>
<tr>
<td>7</td>
<td>40/F</td>
<td>7x6</td>
<td>5x5</td>
<td>4x3</td>
<td>1.316</td>
<td>0.559</td>
</tr>
<tr>
<td>8</td>
<td>52/M</td>
<td>6x6</td>
<td>6x4</td>
<td>3x3</td>
<td>2.230</td>
<td>0.482</td>
</tr>
<tr>
<td>9</td>
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<td>4x3</td>
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<td>0.119</td>
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<td>10</td>
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<td>0.577</td>
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<tr>
<td>11</td>
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<td>5x5</td>
<td>n.d.</td>
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<td>0.706</td>
</tr>
<tr>
<td>12</td>
<td>63/F</td>
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<td>2x3</td>
<td>0.789</td>
<td>0.102</td>
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<tr>
<td>13</td>
<td>28/M</td>
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<td>5x3</td>
<td>4x4</td>
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<td>1.041</td>
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<tr>
<td>14</td>
<td>24/M</td>
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<td>5x5</td>
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<td>0.892</td>
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<tr>
<td>15</td>
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<tr>
<td>16</td>
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<td>n.d.</td>
<td>0.471</td>
<td>0.493</td>
</tr>
<tr>
<td>17</td>
<td>23/F</td>
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<td>5x4</td>
<td>2x2</td>
<td>0.704</td>
<td>0.590</td>
</tr>
<tr>
<td>18</td>
<td>57/F</td>
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<td>5x4</td>
<td>0.547</td>
<td>0.508</td>
</tr>
<tr>
<td>19</td>
<td>22/F</td>
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<td>5x4</td>
<td>4x4</td>
<td>0.700</td>
<td>0.417</td>
</tr>
<tr>
<td>20</td>
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<td>4x5</td>
<td>n.d.</td>
<td>0.565</td>
<td>0.533</td>
</tr>
<tr>
<td>21</td>
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<td>5x6</td>
<td>n.d.</td>
<td>1.631</td>
<td>1.046</td>
</tr>
<tr>
<td>22</td>
<td>40/F</td>
<td>6x5</td>
<td>7x6</td>
<td>n.d.</td>
<td>3.161</td>
<td>1.137</td>
</tr>
<tr>
<td>23</td>
<td>48/M</td>
<td>5x5</td>
<td>3x3</td>
<td>5x3</td>
<td>0.407</td>
<td>0.431</td>
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<tr>
<td>24</td>
<td>62/F</td>
<td>8x4</td>
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<td>n.d.</td>
<td>0.636</td>
<td>0.416</td>
</tr>
<tr>
<td>25</td>
<td>59/M</td>
<td>7x5</td>
<td>5x5</td>
<td>5x5</td>
<td>0.932</td>
<td>0.587</td>
</tr>
<tr>
<td>C1</td>
<td>28/M</td>
<td>5x4</td>
<td>1x1</td>
<td>1x1</td>
<td>0.047</td>
<td>0.038</td>
</tr>
<tr>
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<td>1x2</td>
<td>1x1</td>
<td>0.105</td>
<td>0.059</td>
</tr>
<tr>
<td>C3</td>
<td>26/F</td>
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<td>1x1</td>
<td>1x1</td>
<td>0.087</td>
<td>0.052</td>
</tr>
<tr>
<td>C4</td>
<td>22/F</td>
<td>4x3</td>
<td>2x1</td>
<td>2x1</td>
<td>0.029</td>
<td>0.027</td>
</tr>
<tr>
<td>C5</td>
<td>38/M</td>
<td>3x3</td>
<td>1x1</td>
<td>1x1</td>
<td>0.047</td>
<td>0.035</td>
</tr>
</tbody>
</table>

n.d. = patients which are not skin tested with 31 kDa
*specific IgE cut off 0.189 OD
*specific IgE cut off 0.126 OD
Figure 5.3A: Histamine released from stripped basophils re-sensitized with individual patients’ sera (n=15) on challenge with kidney bean extract or purified protein, separately. C1-C5= controls.

Figure 5.3B: Scatter plot of the correlation analysis between % histamine release with kidney bean extract and 31 kDa purified protein.

Hemagglutination assay: Both 31 kDa protein and PHA (Sigma Chemicals, USA) showed positive HA assay as compared to the negative control PBS. Human erythrocytes remain suspended in the wells to form a uniform reddish color across the well due to agglutinating activity of both 31 kDa protein and purified PHA. However, in case of PBS
erythrocytes were settled down at the corner of the plate (Figure 5.4C). The HA titre of 31 kDa was 15.62 µg/ml (Figure 5.4D).

**Mass spectrometric analysis:** The 31 kDa protein resolved as two discrete spots on 2-DE (Figure 5.5). Both the spots on spectrometric analysis were identified as isoforms of ‘erythroagglutinating phytohemagglutinin; PHA-E’. Sequence coverage of both the isoforms were more than 28%. A total of 39 peptides were matched to the database and 21 and 19 peptides showed extensive homology (p<0.05) in the respective isoforms. PHA is a defensin protein and function as host defense protein (Table 5.2).

### Table 5.2: Peptide mass fingerprint database search of selected spots from 2-DE of purified 31 kDa kidney bean proteins.

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Accession</th>
<th>Identification</th>
<th>Matches of peptides</th>
<th>Sequence Coverage (%)</th>
<th>MASCOT score</th>
<th>Mass/pl</th>
<th>Proposed function</th>
</tr>
</thead>
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<td>Full=Erythroagglutinating phytohemagglutinin; AltName: Full=PHA-E</td>
<td>39(21)</td>
<td>32</td>
<td>593</td>
<td>29.7/5.15</td>
<td>defensive protein</td>
</tr>
<tr>
<td>2</td>
<td>P05088</td>
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<td>39(19)</td>
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<td>494</td>
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**Allergenic potency of 31 kDa protein and its cross-reactivity:** The 31 kDa protein showed a dose dependent inhibition of IgE binding with self protein in competitive ELISA. A maximum inhibition of 98% was achieved with 1 µg of 31 kDa protein as inhibitor whereas 50% inhibition of IgE binding was obtained with 102 ng of self protein (31 kDa) as inhibitor.

However, a maximum inhibition of 61% was observed in IgE binding to crude kidney bean extract (solid phase) when 1 µg of 31 kDa protein was used as an inhibitor. Whereas, for 50% IgE inhibition to crude kidney bean extract, 976 ng of purified protein was required as inhibitor. For 50% IgE inhibition to solid phase crude kidney bean extract, only 67.3 ng of self protein extract was required (Figure 5.6).

Cross-reactivity of 31 kDa protein was assessed with black gram, peanut and pigeon pea by ELISA inhibition using serum pools from patients SPT positive only to kidney bean
extract and negative to the tested cross-reactive antigen. Here, purified 31 kDa protein from kidney bean was coated on solid phase and different legume extracts (kidney bean, black gram, peanut and pigeon pea) were used as inhibitors. Fifty six ng of kidney bean protein (inhibitor) was required for 50% inhibition of IgE binding to solid phase 31 kDa in ELISA, whereas peanut, black gram and pigeon pea caused same inhibition with 185, 228 and 1300 ng of protein(s), respectively (Figure 5.6).

Figure 5.4: (A) Glycoprotein detection by Periodic Acid Schiff’s (PAS) staining. Lane 1 = kidney bean extract, lane 2 = purified 31 kDa protein. (B) Immunoblot analysis of purified protein after periodate treatment. The 31 kDa protein was electro-transfered onto nitrocellulose membrane and then periodate oxidation was done. The strip was washed, blocked with 3% defatted milk and immunoblotted with pooled patients’ sera. Lane 1: 31 kDa protein (untreated), lane 2: 31 kDa protein after periodate treatment. (C) Hemagglutination assay of 31 kDa protein, PHA (positive control) and PBS (negative control). Both 31 kDa and PHA form uniform reddish color across the well with a minimum concentration of 15.62 μg/ml. (D) Agglutination of 31 kDa and PHA protein using human erythrocytes.
Figure 5.5: 2DE gel electrophoresis of purified 31 kDa protein showing two close spots (1 & 2) at the same molecular weight. Both the spots were identified as PHA-E after mass spectrometric analysis.

Figure 5.6: IgE ELISA inhibition of kidney bean extract and 31 kDa protein using self protein and different legumes as inhibitor. Kidney bean positive patients’ pooled sera (1:10 v/v) was preincubated with 5, 10, 50, 100, 1000, 10000 ng of inhibitor. ELISA was carried out on solid phase coated kidney bean extract (2 µg/100µl/well) or 31 kDa protein (500 ng/100µl/well) and preincubated sera.
Chapter 5

Purification and Characterization of 31 kDa Kidney Bean Allergen

Enzymatic digestion of purified protein: Enzymatic digestion of purified 31 kDa kidney bean protein was assessed by SGF. The 31 kDa protein remained undigested (stable) even after 1 h treatment in SGF (Figure 5.7). The negative control, BSA was digested completely by SGF within 1 min and positive control, lactoglobulin remained stable to digestion even after 1 h of treatment.

Figure 5.7: Simulated gastric fluid (SGF) digestion of 31 kDa protein. M=molecular weight marker, Lane 1: 31 kDa protein (undigested); lanes 2-8: 31 kDa protein incubated in SGF for 0.5, 1, 5, 10, 15, 30 and 60 min; lane 9: pepsin. The protein (digest) was electrophoresed on SDS-PAGE and visualized by CBB staining.

DISCUSSION

Legumes are consumed worldwide and India is one of the major consumers. Studies on food allergens are very limited and restricted to only few foods such as egg, milk, cereals in Indian population (Parihar et al., 1984; Kumar et al., 2007; Sharman et al., 2000). Legumes such as peas, peanut, lentils, black gram, soy bean and chickpeas have been associated with allergic reactions (Kumari et al., 2006; Mittag et al., 2004; Zoccatelli et al., 2010; Akkerdaas et al., 2012). Presently, whole mass extracts are used in the allergic diagnosis. But their use has several pitfalls like batch to batch variation, low expression or concentration of many important allergens in whole extracts. Apart from this many endogenous enzymes may degrade allergens during extraction procedures. However, these problems can be overcome by using purified proteins for diagnosis termed as component resolved diagnosis (CRD) (Hoffmann-Sommergruber et al., 2008).
Many protein allergens have been purified and characterized which have proved promising in selective diagnosis/therapy of allergy. Marsh et al. (2008) purified native peanut allergens Ara h 1, 2, 3, 4 and 6 for diagnosis of peanut allergy. Ara h 1 (63 kDa) was purified on a ConA Sepharose-4B affinity column after ammonium sulfate precipitation. Ara h 2 and 6 were purified after ammonium sulfate precipitation and gel filtration followed by anion exchange and reverse-phase HPLC. Ara h 3/4 were purified following anion exchange and gel filtration chromatography. A major allergen from soy (Gly m Bd), was purified using mAb as a ligand (Tsujii et al., 1997). Anion-exchange chromatography and reverse-phase HPLC were also used to purify Len c 1.01- a major allergen from lentil (López-Torrejón et al., 2003). Two major globulins, α-conglutin (11S and “legumin-like”) and β-conglutin (7S and “vicilin-like”) and two minor globulins, γ-conglutin and δ-conglutin from lupin (Lupinus albus) were purified using centrifugation and isolation by anion-exchange chromatography followed by size-exclusion chromatography (Nadal et al., 2011). A seed storage 24 kDa protein was purified from the seeds of Lathyrus sativus by ammonium sulfate fractionation and ion-exchange chromatography (Qureshi et al., 2006). Another 25 kDa legume protein from Vigna unguiculata extract was fractionated using ammonium salt precipitation followed by gel chromatographic separation (Chanana et al., 2004). In the present study, we have used combination of anion exchange chromatography, gel filtration and reverse-phase HPLC on C18 column to get purified 31 kDa kidney bean allergen.

An allergen is defined by its ability to exhibit IgE binding through its epitope and cross-linking already bound IgE on IgE/FceRI receptor on the surface of mast cells and basophils and subsequent mediator release like histamine (Kay, 2002). In the present study, IgE binding of the purified 31 kDa protein was assessed under in vivo (SPT), in vitro (ELISA and immunoblotting) and ex vivo (histamine release assay) conditions. The allergic relevance of purified 31 kDa protein was exhibited by IgE binding with more than 88% of kidney bean sensitive patient's sera. In addition, 78% kidney bean sensitive patients showed SPT positive reaction to purified protein. It inhibited up to 73% IgE binding in ELISA inhibition with 10 μg of purified protein as inhibitor and kidney bean on solid phase.
The interaction of allergen with IgE bound to FcɛRI on the surface of mast cells' basophils triggers the release of histamine and other biological mediators responsible for the clinical allergic symptoms (Kay et al., 2001). A study showed that mediator release from basophil significantly correlated with airway reactivity in asthmatic patients (Gaddy et al., 1986) highlighting the effector function of basophils in inflammatory diseases and the pathologic significance of mediator release. We observed a significant histamine release from kidney bean positive patients’ samples (n=15) as compared to controls after 31 kDa protein challenge. This indicated that purified protein is a potent allergen having clinical relevance in diagnosis. Specific IgE values and histamine release with purified 31 kDa protein and kidney bean extract correlated significantly (p<0.01). In an earlier study by Kumari et al. (2012), 7/9 black gram sensitive patients were found SPT positive to its 28 kDa purified protein whereas, 77% black gram patients showed significant IgE binding. Lopez-Torrejon et al. (2003) showed IgE binding with ≥80% of the lentil patient’s sera to purified Len c 1.01.

In my study, a total of 102 ng of 31 kDa protein was required for 50% inhibition of self protein whereas 976 ng of 31 kDa protein was required for kidney bean extract suggesting it as a potent allergen. Cross-reactivity has been reported previously among legume allergens (Ibáñez et al., 2003; Kumari et al., 2006; Verma et al., 2012). In the present study, 56 ng of kidney bean extract was required to obtain 50.0% inhibition of IgE binding on solid phase 31 kDa, whereas 185 and 228 ng of peanut and black gram were required, respectively, for the same. In another study, inhibition of 65% IgE binding was observed with 2 μg of purified lentil protein as inhibitor and whole lentil extract on solid phase in immunoblot inhibition (López-Torrejón et al., 2003). A major peanut allergen Ara h1 caused 50% inhibition at 5.5 ng as compared to 1.5 ng of the self-extract.

Resistance to proteolytic enzymes and heat is a property associated with food allergens. Many food allergens or stable allergen fragments have been shown to resist conditions of the gastrointestinal tract, and thus have the potential to sensitize the immune system. In the present study, pepsin digestion of 31 kDa protein showed no cleavage products in the SDS-PAGE. It suggested that the protein is available to be taken up by the immune cells
The purified 31 kDa protein is identified as PHA-E after mass spectrometric analysis which is a defensive protein. This protein is identified as a glycoprotein. Periodate oxidation does not reduce the IgE binding of 31 kDa protein immunoblot. Thus, ruling out the possibility of any non-specific binding due to the presence of carbohydrate. The major food allergens identified as class 1 allergens are water-soluble 10 to 70 kDa glycoproteins such as caseins (milk), ovomucoid (egg), nonspecific lipid transfer proteins like Mal d 3 (apple) and Zea m 14 (corn) (Breitenede et al., 2005). Among legumes, Ara h 1, a 65 kDa major allergen is a glycoprotein belonging to the vicilin family (Shin et al., 1998). Len c 1 from lentil also showed 3 isoforms varying in their degree of N-glycosylation (Lopez-Torrejon et al., 2003). The 31 kDa protein in the present study showed agglutination activity similar to commercial PHA obtained from Sigma.

In conclusion, the 31 kDa kidney bean protein is purified, identified as phytohemagglutinin and have potential for therapy in kidney bean allergy.