Chapter 3:

Effect of processing on legume allergenicity
INTRODUCTION

Legumes are important dietary components and are rich source of proteins used in bakery, confectionery and snacks all over 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; Rougé et al., 2011). The most common legumes of allergenic significance are peanut (Arachis hypogaea), soybean (Glycine max), lentil (Lens culinaris), chick pea (Cicer arietinum), kidney bean (Phaseolus vulgaris), black gram (Vigna mungo) and pigeon pea (Cajanus cajan) (Ibáñez Sandin et al., 1999; Cordie, 2004; Sicherer et al., 2006; Kumari et al., 2006; Kasera et al., 2011). Most food allergens can cause reactions when ingested either in the raw form or after being cooked or even digested, but some allergens, such as those in fruits and vegetables, cause allergic reactions primarily if eaten raw. Cross-reactivity can occur when a food allergen has structural or sequence similarity with a different food allergen or aeroallergen (Burks et al., 2012).

Severity of allergic reactions varies based on the amount of food ingested, co-ingestion of other foods, and preparation of the food (cooked, raw, or processed) (Boyce et al., 2010). It can be influenced by the patient’s age, as well as rapidity of absorption, which can be influenced by whether the food was eaten on an empty stomach or close to a time of exercise. The presence of other co-morbid conditions, such as asthma or atopic dermatitis, can also influence severity. Food-induced anaphylaxis is a serious allergic reaction that is rapid in onset and can cause death (Burks et al., 2012). Survey data from the United States shows that children with food allergy have a 4, 2.4 and 3.6 fold increased likelihood of having asthma, atopic dermatitis and respiratory allergies, respectively, compared with children without food allergy (Branum and Lukacs, 2009).

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 as the patient is always at risk of accidental exposure. Systemic SCIT has been investigated in the past but it resulted in significant adverse effects (Oppenheimer et al., 1992). Alternative forms of therapy have been sought to provide systemic treatment with reduced risk and side effects. For a variety of food allergens, OIT is effective in reducing...
clinical reactivity in some patients, but its ability to induce tolerance still remains uncertain. Diets containing extensively heated (baked) milk and egg might represent an alternative approach to OIT, however, further studies of this approach are necessary. Hence, the approach that could reduce the allergenicity without altering the nutritional value of food 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 reduces the antigenicity of ovalbumin, bovine serum albumin and milk protein and shrimp tropomyosin (Kume et al., 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 et al., 1996; Besler et al., 2001). Later, a combination of γ-radiation 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 Sokorai, 2008; Bhat et al., 2008; Nour et al., 2009). The present study was therefore aimed to determine the effect of thermal processing, γ-irradiation or both on stability of allergenic proteins of kidney bean, black gram and peanut.
MATERIAL AND METHODS

The methods followed to study the above objectives are illustrated in the following pages and also with the flow chart Figure 3.1.

Processing of food materials

Thermal processing: Whole raw legume seeds of kidney bean, black gram and peanut were subjected to boiling at 121°C or 15 pounds per square inch (psi) for 15 min. The boiled seeds were freeze dried and powdered using mortar and pestle.

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
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Medicine and Allied Sciences (INMAS), Delhi. The source of γ-irradiation used in the present study was Cobalt-60.

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 min and then subjected to γ-irradiation (25 kGy). The boiled seeds were freeze dried and powdered using mortar and pestle.

The materials from unprocessed legume seeds without thermal or γ-irradiation were used as control for assessment of allergenicity.

Preparation of antigenic extracts: The extraction was carried out following the protocol as described earlier in chapter 2. Briefly, seed materials after crushing were defatted in diethyl ether at 4° C. Antigen extraction was carried out in 1:20 w/v ammonium bicarbonate buffer (50 mM, pH 8.0) by continuous stirring for 6 h at 4° C. The extracts were centrifuged at 10,000 g and the supernatant was filtered through a 0.22 μm membrane 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).

Processing of raw extracts

Thermal processing: Raw extracts of study materials were subjected to boiling at 121° C (15 psi) for 15 min. Boiled raw extracts were centrifuged and precipitated proteins were used for allergenicity testing. The precipitated protein(s) were resuspended in urea (0.05M) - thiourea (0.01M) buffer for complete solubilization.

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 extracts resuspended in urea/thiourea buffer were used as control in allergenicity assessment of precipitated proteins.
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Study subjects: The study included patients of allergic rhinitis and asthma (mean age 30.7±13.9) with confirmed history of sensitization to the three legumes, kidney bean, black gram and peanut. The study protocol was approved by Institutional Human Ethics Committee. Informed written consents were obtained from all the patients and controls for participation in the study. SPT was performed with raw legume seed extracts as described earlier 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. IgE binding in ELISA was also elucidated using various processed antigens.

IgE inhibition assay: The allergenic potential of raw and processed legume seed extracts were determined by ELISA inhibition (competitive ELISA) using hypersensitive pooled patients' sera. Legume seed extract (1 µg/100 µl per well) was coated in carbonate buffer overnight at 4° C in a microtitre plate. The patients' pooled serum (1:10 v/v) for respective antigen was preincubated with 10, 50, 100, 500, 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

\[
\frac{1}{\text{OD of sample with inhibitor}} - \text{OD of sample without inhibitor} \times 100
\]

Competitive ELISA was also performed using differently processed antigens as inhibitor and raw extract on solid phase to elucidate the potential of each processed antigen in comparison to their respective raw extract.

SDS-PAGE and Immunoblot: To study the protein profile, extracts from processed and unprocessed legumes (25 µg protein per lane) were resolved on 12% reducing gel (Singh et al., 2006). The proteins separated on SDS-PAGE were stained with CBB.

For immunoblot, the resolved proteins were transferred onto NCM and processed as described earlier in chapter 2.
Simulated gastric fluid (SGF) digestion: The digestibility of the processed and unprocessed legume proteins was examined in the SGF, as described by Kumari et al. (2012). Briefly, a protein extract (680 µg) was treated with 200 µL of prewarmed SGF (US Pharmacopeia) containing 0.32 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. These aliquots were quickly mixed with 26 µL of a sample buffer (containing 2% β-mercaptoethanol and 4% SDS) for SDS-PAGE together with 6 µL of Na2CO3 solution (200 mmol/L). The mixture was then boiled for 5 minutes and stored at ~20° C until further analyses. As control, each protein sample was treated with SGF that did not contain pepsin A and processed as described above. The digestibility of two known allergens namely, milk lactoglobulin and BSA, was examined to assess the activity of the SGF. These two purified proteins were purchased from Sigma Chemicals, USA.

Statistical analysis: Values are represented as mean±SD. Statistical significance was calculated using one way analysis of variance using software Epi Info 3.3.2. and SISA. The significance level was considered to be p<0.05.

RESULTS

Soluble protein concentration of legumes: Boiling at 121° C for 15 min led to a 3-4 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 (0.95±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 powder and even in the raw extracts of the three legumes as the protein concentration in the antigenic extract was same in irradiated and untreated 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 raw extracts. There was no significant difference in the protein
concentration among boiled and boiled+γ-irradiated legume seed extracts (p>0.05) (Figure 3.2).

![Graph showing protein concentration](image)

**Figure 3.2**: Effect of processing on the soluble protein concentration of kidney bean, black gram and peanut extracts.

**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 study legumes. 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 60-90% (mean 83±11.6%) (p<0.0001) and in peanut it 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 3.1, Figure 3.3). 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±7.9%) as compared to their respective raw antigens (p<0.001) (Table 3.1, Figure 3.3). 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 protein (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 3.1, Figure 3.3). The IgE binding to boiled+γ-irradiated protein (precipitate) was reduced further in kidney bean (44-64%, mean 53±5.9%) (p=0.039), black gram (56-96%, mean 79±12.3%) (p<0.001) and peanut (43-68%, mean 52±8.4%) (p=0.006) as compared to their respective raw antigens (Table 3.1, Figure 3.3).

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.

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 3.2, Figure 3.4A, B and 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 3.2, Figure 3.4A, B and C).

Boiling also brought a reduction in allergenic potential of precipitated protein of kidney bean (6 folds), black gram (4 folds) and peanut (8 folds) which was also significant (p<0.001) (Table 3.3, Figure 3.5A, B and 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 fraction than the single processing by boiling (p<0.001) (Table 3.3, Figure 3.5A, B and C).
Figure 3.3: Specific IgE against raw, boiled soluble (BS), boiled soluble+\(\gamma\)-irradiated (BS+\(\gamma\)), boiled precipitate (BP) and boiled precipitate+\(\gamma\)-irradiated (BP+\(\gamma\)) 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+\(\gamma\), raw vs BP+\(\gamma\).
### Table 3.1: Percent reduction in specific IgE binding of processed legume extracts by ELISA.

**Kidney bean**

<table>
<thead>
<tr>
<th>Patients(1)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS</td>
<td>83±6.4</td>
<td>72±5.2</td>
<td>61±5.3</td>
<td>70±6.7</td>
<td>77±4.2</td>
<td>77±9.5</td>
<td>75±6.8</td>
<td>82±9.5</td>
<td>71±4.6</td>
<td>73±7.1</td>
<td>74.0±6.5</td>
</tr>
<tr>
<td>BS+γ</td>
<td>93±4.6</td>
<td>93±8.3</td>
<td>85±3.7</td>
<td>87±6.7</td>
<td>85±3.9</td>
<td>89±5.8</td>
<td>92±7.3</td>
<td>99±6.2</td>
<td>86±6.1</td>
<td>91±6.9</td>
<td>89±8.3</td>
</tr>
<tr>
<td>BP</td>
<td>46±5.3</td>
<td>31±3.3</td>
<td>27±6.5</td>
<td>36±7.8</td>
<td>32±4.1</td>
<td>37±3.7</td>
<td>29±3.9</td>
<td>36±4.1</td>
<td>34±4.5</td>
<td>33±6.8</td>
<td>34±5.3</td>
</tr>
<tr>
<td>BP+γ</td>
<td>59±2.9</td>
<td>50±6.9</td>
<td>44±6.9</td>
<td>57±8.8</td>
<td>60±5.9</td>
<td>64±4.8</td>
<td>48±7.7</td>
<td>50±5.7</td>
<td>54±3.3</td>
<td>53±9.6</td>
<td>52±6.9</td>
</tr>
</tbody>
</table>

**Black gram**

| BS | 60±6.9 | 80±6.3 | 86±6.8 | 90±6.5 | 92±7.1 | 91±6.9 | 85±6.6 | 84±5.2 | 93±7.4 | 67±4.9 | 72±5±1.6 |
| BS+γ | 61±3.9 | 84±6.3 | 90±4.7 | 92±5.8 | 95±6.1 | 95±9.1 | 90±8.7 | 90±6.4 | 99±4.9 | 72±4.4 | 86±6±11.6 |
| BP | 41±7.3 | 60±4.8 | 80±6.9 | 78±6.5 | 86±4.9 | 82±7.6 | 78±6.0 | 79±5.3 | 95±6.4 | 61±3.9 | 72±5±16.6 |
| BP+γ | 56±6.9 | 74±7.5 | 83±4.6 | 83±9.3 | 89±8.8 | 87±5.4 | 82±6.2 | 82±6.7 | 96±4.6 | 61±3.8 | 79±3±12.3 |

**Peanut**

| BS | 58±3.7 | 54±5.7 | 78±4.5 | 60±6.3 | 63±5.6 | 71±6.4 | 58±7.2 | 57±6.5 | 65±3.9 | 58±7.1 | 62±8±7.2 |
| BS+γ | 65±3.6 | 68±7.4 | 87±8.5 | 69±3.9 | 69±3.7 | 87±6.7 | 65±7.4 | 72±5.3 | 72±4.6 | 73±5.7 | 72±7±9.7 |
| BP | 46±5.2 | 41±5.7 | 63±6.3 | 49±4.7 | 46±4.7 | 57±4.1 | 43±6.2 | 28±3.8 | 44±5.6 | 28±2.6 | 44±4±11.1 |
| BP+γ | 50±6.2 | 45±3.6 | 68±4.7 | 52±5.9 | 55±3.3 | 64±7.3 | 50±4.2 | 43±2.6 | 52±6.8 | 43±3.1 | 52±8±8.4 |

BS= boiled soluble protein
BS+γ= boiled soluble protein followed by γ-irradiation
BP= boiled precipitated protein
BP+γ= boiled precipitated protein followed by γ-irradiation

\(1\) Patients with significantly high specific IgE (O.D.) i.e. ≥3 as compared to normal human sera (0.08) were selected.
A significant change in the allergenic potential (IC₅₀) was not 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 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.

Table 3.2: IC₅₀ of soluble protein of processed and raw legume extracts by ELISA inhibition.

<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±6.56</td>
<td>328±34.77</td>
<td>34±6.00</td>
</tr>
<tr>
<td>γ-irr25 kGy</td>
<td>98±6.00</td>
<td>320±13.86</td>
<td>32±3.61</td>
</tr>
<tr>
<td>Boiled 121° C</td>
<td>715±24.02</td>
<td>920±33.60</td>
<td>883±19.16</td>
</tr>
<tr>
<td>Boiled 121° C+γ-irr25 kGy</td>
<td>1000±14.73</td>
<td>1000±30.79</td>
<td>1600±48.54</td>
</tr>
</tbody>
</table>

Table 3.3: IC₅₀ of precipitated protein of processed and raw legume extracts by ELISA inhibition.

<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>67±3.22</td>
<td>193±8.87</td>
<td>100±6.67</td>
</tr>
<tr>
<td>Boiled</td>
<td>434±13.76</td>
<td>800±31.32</td>
<td>840±28.34</td>
</tr>
<tr>
<td>Boiled+γ-irr25 kGy</td>
<td>887±23.49</td>
<td>220±57.32</td>
<td>2330±62.65</td>
</tr>
</tbody>
</table>
Figure 3.4: (A) ELISA inhibition with raw and various processed kidney bean as inhibitor. Kidney bean patients’ pooled sera were preincubated separately with 10, 50, 100, 1000, 10000 ng of raw and various processed kidney bean extracts as inhibitors. ELISA was carried out on solid phase coated with raw kidney bean extract and preabsorbed sera. (B) ELISA inhibition with raw and various processed black gram as inhibitor. Black gram positive patients’ pooled sera were preincubated separately with 10, 50, 100, 1000, 10000 ng of raw and various processed black gram extracts as inhibitors. ELISA was carried out on solid phase coated with raw black gram extract preabsorbed sera. (C) ELISA inhibition with raw and various processed peanut as inhibitor. Peanut positive patients’ pooled sera were preincubated separately with 10, 50, 100, 1000, 10000 ng of raw and various processed peanut extracts as inhibitors. ELISA was carried out on solid phase coated with raw peanut extract and preabsorbed sera.
Figure 3.5: (A) ELISA inhibition with raw and precipitated kidney bean protein after processing as inhibitor. Kidney bean positive patients' pooled sera were preincubated separately with 10, 100, 500, 1000, 10000 ng of raw and various processed kidney bean extracts as inhibitors. ELISA was carried out on solid phase coated with raw kidney bean extract and preabsorbed sera.

(B) ELISA inhibition with raw and precipitated black gram protein after processing as inhibitor. Black gram positive patients' pooled sera were preincubated separately with 10, 100, 500, 1000, 10000 ng of raw and various processed black gram extracts as inhibitors. ELISA was carried out on solid phase coated with raw black gram extract and preabsorbed sera.

(C) ELISA inhibition with raw and precipitated peanut protein after processing as inhibitor. Peanut positive patients' pooled sera were preincubated separately with 10, 100, 500, 1000, 10000 ng of their respective raw and various processed peanut extracts as inhibitors. ELISA was carried out on solid phase coated with raw peanut extract and preabsorbed sera.
Effects of processing on legume allergenicity

Changes in protein profile of legumes after thermal and non-thermal processing

SDS-PAGE resolved raw kidney bean extract into 22 protein bands of 14-150 kDa (Figure 3.6A, lane 1), boiled kidney bean into 13 protein bands of 14-150 kDa (Figure 3.6A, lane 7), re-suspended precipitated protein of kidney bean after thermal processing resolved in 11 protein bands of 14-97 kDa (Figure 3.6A, lane 9). γ-irradiation alone did not alter the protein profile of kidney bean seed extract even at highest dose of 25 kGy (Figure 3.6A, lane 2-6). Thermal processing in combination with γ-irradiation reduced number of visible protein bands to 7 in kidney bean (Figure 3.6A, lane 8). The re-suspended precipitated protein of kidney bean after combination processing resolved in 4 protein bands (Figure 3.6A, lane 10).

![Figure 3.6: SDS-PAGE protein profile of kidney bean (A), black gram (B) and peanut (C)](image)

Lane 1: Raw, lane 2: Irradiated 5 kGy, lane 3: Irradiated 10 kGy, lane 4: Irradiated 15 kGy, lane 5: Irradiated 20 kGy, lane 6: Irradiated 25 kGy, lane 7: boiled at 121° C for 15 min, lane 8: boiled+γ-irradiated, lane 9: boiled precipitate and lane 10: boiled+γ-irradiated precipitate respectively. M: Molecular weight markers.

SDS-PAGE resolved raw black gram into 20 protein bands of 12-120 kDa (Figure 3.6B, lane 1), boiled black gram into 12 protein bands of 15-120 kDa (Figure 3.6B, lane 7). The re-suspended precipitated protein of black gram after thermal processing resolved in 8 protein bands (Figure 3.6B, lane 9). γ-irradiation did not alter the protein profile of black gram even at highest dose of 25 kGy (Figure 3.6B, lane 2-6). Thermal processing in combination with γ-irradiation reduced number of visible protein bands to 7 in black gram (Figure 3.6B, lane 8). The re-suspended precipitated protein of black gram extract after combination processing resolved in 8 protein bands (Figure 3.6B, lane 10).
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SDS-PAGE resolved raw peanut into 21 protein bands of 13-110 kDa (Figure 3.6C, lane 1), boiled peanut into 12 protein bands of 11-72 kDa (Figure 3.6C, lane 7). The re-suspended precipitated protein of black gram and peanut after thermal processing resolved in 8 protein bands (Figure 3.6C, lane 9). ɣ-irradiation did not alter the protein profile of black gram even at highest dose of 25 kGy (Figure 3.6C, lane 2-6). Thermal processing in combination with ɣ-irradiation reduced number of visible protein bands to 8 in peanut (Figure 3.6C, lane 8). The re-suspended precipitated protein of peanut extract after combination processing resolved in 6 protein bands (Figure 3.6C, lane 10).

Changes in allergenic profile of legume proteins by thermal processing and ɣ-irradiation

The IgE binding components in kidney bean, black gram and peanut extracts were analyzed by Western blot using pooled sera of hypersensitive patients to the respective antigen. The allergenic protein profile of all three legumes remained same to that of unprocessed seed materials after different dose treatment (5, 10, 15, 20 and 25 kGy) of ɣ-irradiation (Figure 3.7A, B and C [lane 2-6]). However changes in protein profile were observed after boiling and boiling followed by ɣ-irradiation.

![Western Blot Images](image-url)

Figure 3.7: Allergenic protein profile of kidney bean (A), black gram (B) and peanut (C) by western blot. Lane 1: Raw, lane 2: Irradiated 5 kGy, lane 3: Irradiated 10 kGy, lane 4: Irradiated 15 kGy lane 5: Irradiated 20 kGy, lane 6: Irradiated 25 kGy, lane 7: boiled at 121°C for 15 min, lane 8: boiled+ɣ-irradiated, lane 9: boiled precipitate and lane 10: boiled+ɣ-irradiated precipitate respectively. M: Molecular weight markers.
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Unprocessed (raw) kidney bean extract showed 15 IgE-binding proteins of 120, 95, 70, 58, 55, 50, 45, 42, 40, 37, 34, 26, 24, 18 and 16 kDa (Figure 3.7A, lane 1). Boiled kidney bean showed 12 IgE binding proteins ranging from 14-70 kDa (Figure 3.7A, lane 7). The precipitated protein of kidney bean after thermal processing showed 5 IgE binding protein fractions in immunoblot (Figure 3.7A, lane 9). Thermal processing in combination with γ-irradiation showed 6 allergenic proteins in kidney bean (Figure 3.7A, lane 8). The precipitated protein of kidney bean after thermal+γ-irradiation processing showed 3 IgE binding protein fractions in immunoblot (Figure 3.7A, lane 10).

Unprocessed (raw) black gram (raw) showed 10 IgE binding proteins of 102, 90, 59, 57, 54, 44, 34, 29, 20 and 17 kDa (Figure 3.7B lane 1). Boiled black gram showed 4 IgE binding proteins of 44-59 kDa (Figure 3.7B, lane 7). The precipitated protein of black gram after thermal processing showed 4 IgE binding protein fractions on immunoblot. Thermal processing in combination with γ-irradiation showed 3 allergenic proteins in black gram (Figure 3.7B, lane 8). The precipitated protein of black gram showed 5 and 4 bands respectively after single and combination processing (Figure 3.7B, lane 9-10).

Boiled peanut showed 13 proteins of 14-64 kDa (Figure 3.7C, lane 7). The precipitated protein of peanut after thermal processing showed 8 IgE binding protein fractions in immunoblot. Thermal processing in combination with γ-irradiation showed 13 IgE binding protein fractions (Figure 3.7C, lane 8). The precipitated protein of peanut showed 7 bands after single and combination processing (Figure 3.7C, lane 9-10).

Changes in enzymatic digestion of legume proteins

Kidney bean: Most of the kidney bean proteins (≥58 kDa) from unprocessed seed materials were digested by the SGF within 5 minutes. However, a number of protein fractions ranging from 16-55 kDa were stable to pepsin digestion after 1 h (Figure 3.8A).

Proteins of 24, 37, 58 and 85 kDa which were easily digestible in raw kidney bean seed materials became resistant to digestion after thermal processing. Nearly all of the heat stable protein fractions were stable to SGF digestion (Figure 3.8B).
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Processing of kidney bean by γ-irradiation did not alter the digestibility of the proteins in SGF. The proteins remained equally susceptible or resistant to digestion as in unprocessed seed materials (Figure 3.8C). Boiling+γ-irradiation in combination increased the digestibility of proteins in SGF since only two proteins of 34 and 58 kDa remained undigested after one hour in SGF (Figure 3.8D).

Black gram: A majority of raw black gram proteins were stable to SGF digestion but a few were partially digested with increase in digestion time. However, they did not get digested completely even after one hour of SGF treatment (Figure 3.9A). Thermal processing could not change the digestibility of black gram proteins even after one hour in SGF (Figure 3.9B).

Processing of black gram by γ-irradiation alone or boiling + γ-irradiation did not alter the digestibility of the proteins in SGF. The proteins remained equally susceptible or resistant to digestion like unprocessed seed materials (Figure 3.9C, Figure 3.9D).

Peanut: A majority of the raw peanut proteins were digested in less than 5 minutes except three fractions of 14, 17 and 33 kDa which remained stable even after one hour of SGF treatment (Figure 3.10A). Proteins of 13, 26, 29, 39, 46, 50 and 57 kDa which were easily digestible in raw peanut became resistant to digestion after boiling. This shows that the boiling increases the stability of the proteins to digestion (Figure 3.10B).

Processing peanut by γ-irradiation alone did not alter the digestibility of the proteins in SGF (Figure 3.10C). Boiling+γ-irradiation in combination increased the digestibility of peanut proteins in SGF since only 26 kDa protein remained undigested after 1 h of SGF treatment (Figure 3.10D).

However, kidney bean, black gram and peanut protein which precipitates after thermal processing and or combination treatment of thermal+γ-irradiation gets digested by SGF in 5 min which further suggests that processing increases the digestibility of protein and hence reduces allergenicity (Figure 3.8E-F, 3.9E-F, and 3.10E-F respectively).
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Figure 3.8: SDS-PAGE profiles of SGF digest of kidney bean raw (A), boiled at 121° C for 15 min (B), γ-irradiated at 25 kGy (C), boiled+γ-irradiated (D), boiled precipitate (E) and boiled+γ-irradiated precipitated. Lane 1: undigested, lane 2-8 treated for 0.5, 1, 5, 15, 30, 45, 60 min in SGF, lane 9: pepsin, M: Molecular weight markers.
Figure 3.9: SDS-PAGE profiles of SGF digest of black gram raw (A), boiled at 121° C for 15 min (B), γ-irradiated at 25 kGy (C), boiled+γ-irradiated (D), boiled precipitate (E) and boiled+γ-irradiated precipitated. Lane 1: undigested, lane 2-8 treated for 0.5, 1, 5, 15, 30, 45, 60 min in SGF, lane 9: pepsin, M: Molecular weight markers.
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Figure 3.10: SDS-PAGE profiles of SGF digest of peanut raw (A), boiled at 121°C for 15 min (B), γ-irradiated at 25 kGy (C), boiled+γ-irradiated (D), boiled precipitate (E) and boiled+γ-irradiated precipitated. Lane 1: undigested, lane 2-8 treated for 0.5, 1, 5, 15, 30, 45, 60 min in SGF, lane 9: pepsin, M: Molecular weight markers.
DISCUSSION

The plant and animal food allergens belong primarily to a few protein families suggesting that certain conserved structure plays a key role in determining allergenic properties. In addition, the level of exposure and the physicochemical properties of an allergen contributes 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 families like storage or defense related proteins, its abundance and stability to processing and digestion (Breiteneder et al., 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 defences (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 using immunobiochemical methods.

It has been hypothesized that boiling cause protein aggregation leading loss in protein solubility. However Su et al. (2004) did not find any change in the qualitative protein profile of almond, cashew nut and walnut by γ-irradiation alone or in combination with autoclaving, blanching, frying, microwaving or roasting. Further, Zhenxing et al. (2007) reported that processing by irradiation alone or in combination with heat, produces minimal change in SDS-PAGE protein profile and IgE recognition of shrimp allergens. In my study, no qualitative change is observed in the protein profile of study legumes with γ-irradiation alone. But thermal processing or combination of heat+γ-irradiation produced significant change in protein profile since 60-70% of protein bands could not be detected (disappeared) after processing. The combination of heat+γ-irradiation showed a synergistic effect as less protein bands were detected than heat treated or γ-irradiated legume materials, separately.

The extent of loss of protein solubility depends on the type, severity and duration of processing (Zhenxing et al., 2007). In the present study, boiling reduced the soluble protein content 3-4 fold in three selected legumes. Boiling significantly reduced specific IgE binding to kidney bean, black gram and peanut in ELISA as compared to their
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respective raw extracts using individual patient sera. Also a 3-26 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 when γ-irradiation treatment was given individually. This may be attributed to irradiation effect that ionizes molecules at random positions along their trajectories resulting in the release of various radiolytic products (mostly water whose principal radiation products are H+ and OH-) that diffuse in the medium and are responsible for 99.9% of damage to proteins (Kempner et al., 2001). Here the amount of water in boiled seeds was more than dry legume seed powder therefore, the enhanced effect of irradiation was observed on boiled seeds as compared to dry legume seeds. My 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 extracts. On the other hand Kume and Matsuda (1995) demonstrated that 8 kGy irradiation as an effective method for reducing allergenicity of ovalbumin and BSA. Conversely, Leszczyńska 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 reduction 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 Zhenxing et al. (2007) demonstrated 5-30 times increase in IC50 value after heat+γ-irradiation on shrimp allergen. 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 discussed elsewhere. The difference in the results may possibly be due to the fact that in the study by Su et al. (2004) irradiation was followed by autoclaving in contrast to my work 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 protein(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 IgE binding as compared to respective raw legumes but a significant decrease in IgE binding was not observed in case of kidney bean. However, decrease in IgE binding became significant in insoluble kidney bean protein when combination processing method was employed. Also a significant (4-8 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 IgE binding as compared to respective raw legume extracts. Moreover, when combination processing (boiling+γ-irradiation) were employed a further significant (11-23 fold) increase in the IC₅₀ was also observed when compared either to raw or boiled extracts.

The investigation of stability of proteins within the gastrointestinal tract may provide prospective testing for allergenicity and could be a significant and valid parameter that distinguishes food allergens from nonallergens (Wickham et al., 2009). A major characteristic of many food allergens is their resistance to gastric digestion (Lehrer et al., 2002). Studies suggest that food allergens that sensitize patients via the gastrointestinal tract should be able to survive the harsh conditions i.e. acidic and proteolytic environment of the stomach and remain in sufficient intact form to be taken up by the gut and sensitize the mucosal immune system (Taylor and Hefle, 2002; Mills et al., 2004). In the present study, digestibility of two known proteins namely lactoglobulin and BSA was examined in SGF. Lactoglobulin was not digested in SGF even after 1 h whereas BSA was digested within one minute as reported previously (Li et al., 2004). Boiling at 121°C decreased digestibility of kidney bean and peanut proteins and these results are in agreement with an earlier study on peanut (Schmitt and Maleki, 2004). Boiling followed by γ-irradiation (25 kGy) however, increased the digestibility further of both kidney bean and peanut in SGF.

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 allergens. Thus food processing at high temperature followed by γ-
irradiation might be helpful in reducing the risk of food hypersensitive reactions in sensitive individuals. As it becomes sometimes difficult to define the content responsible for causing allergic reaction due to variability in individuals depending upon their sensitivity. In conclusion, thermal processing alone or in combination with γ-irradiation induced significant reduction in allergenic potential of study legumes-kidney bean, black gram and peanut. Hence, both these methods can be employed for preparing hypoallergenic foods for sensitive individuals to reduce the risk of food allergy to legumes.