

RESEARCH ARTICLE

Probing novel allergenic proteins of commonly consumed legumes

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Abstract

Leguminous crops are the main source of protein in Asian subcontinent including India and their proteins may induce allergic reactions in sensitized individuals. Pepsin resistance of proteins is a characteristic feature of most of the allergens. Simulated gastric fluid (SGF) assay as validated by digestion of purified known allergenic and non-allergenic proteins was the basis of this study. Purified allergenic proteins were stable to SGF digestion contrary to rapidly digested non-allergenic proteins. Crude proteins extracts (CPE) of soybean, peanut, chickpea, black gram, kidney bean and Bengal gram were digested *in vitro* to detect their non-digestible proteins. Six proteins from soybean and seven from peanut remained undigested after SGF digestion. Likewise, seven proteins from chickpea (70, 64, 55, 45, 35, 20 and 18 kDa), ten from black gram (47, 30, 29, 28, 26, 24, 22, 16, 14 and 12 kDa), five from kidney bean (45, 29, 24, 20 and 6.5 kDa) and one from Bengal gram (20 kDa) remained undigested in SGF. Most of the proteins stable in SGF for more than 2 min showed similarity with characterized allergens on the basis of their molecular weights as in case of soybean, peanut, chickpea and black gram. Also, soybean and chickpea stable proteins showed IgE binding property with respective allergic patient's sera. The non-digestible proteins from the chickpea, black gram, kidney bean and Bengal gram are being reported for the first time by our group. IgE binding of SGF resistant soybean and chickpea proteins is being reported first time as well.

Keywords: Bengal gram; kidney bean; legume allergy; pepsin resistant proteins; simulated gastric fluid

Abbreviations: BSA: Bovine serum albumin; CPE: Crude Protein Extract; IgE: Immunoglobulin E; Mol Wt: Molecular Weight; SDS-PAGE: Sodium Dodecyl Sulfate-Poly acrylamide Gel Electrophoresis; SGF: Simulated Gastric Fluid; SPT: Skin Prick Test

Introduction

It has been proposed that food allergens that can provoke severe systemic allergic reactions tend to be stable to the peptic and acidic digestion in the gastrointestinal tract.^(1–3) This characteristic of allergenic proteins is mostly tested by analyzing the effects of simulated gastric fluid (SGF) treatment on the integrity and IgE binding capacity of the allergen. Furthermore, resistance to pepsin digestion has been incorporated into the FAO/WHO decision tree approach⁽⁴⁾ and codex alimentarius⁽⁵⁾ guidelines for the allergenicity assessment of genetically modified foods. Stability of proteins to SGF is usually evaluated by means of SDS-PAGE

fractionation, followed by protein staining. Sera of allergic patients were used for specific Immunoglobulin E (IgE) binding after SGF digestion as in case of chestnut and cherry.^(6,7)

Proteins that are resistant to pepsin have an increased probability of reaching the intestinal mucosa and may affect the intestinal immune system.^(1–3) A number of food allergens have been reported to be stable in SGF.^(9,10) Astwood et al.⁽⁹⁾ compared the digestive stability particularly with reference to purified allergenic and non-allergenic food proteins in standard SGF and concluded that most of the non-allergenic proteins degrade within 15 seconds without forming any stable fragments, while some food allergens or parts of it were

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stable in SGF even up to 60 minutes. A number of other studies support the correlation between non-digestibility of proteins in SGF and allergenicity.^(11,12)

Dietary habits of the major population of a region plays an important role in food allergy development i.e., increased consumption of a particular food may lead to increased sensitization to it, e.g., soy allergy in Japan, peanut allergy in the UK, France, and North America and sesame allergy in Israel is common.⁽¹³⁾ Instances of IgE-mediated allergic reactions induced by legumes in Mediterranean and Asian countries have been reported as legumes are an important source of dietary protein in these countries.^(14–16)

Legumes are very important source of food protein in the Indian diet as well. In 2004–05, India produced 30.91 million tonnes of legumes, out of which quantity of pulses was 15.01 million tonnes. This accounts for 26 percent of total production of legumes in the world.⁽¹⁷⁾ The major pulses grown in India are chickpea, pigeon pea, black gram, green gram, kidney bean, Bengal gram, and peas. Therefore, chances of Indians showing sensitization towards these legumes are more. However, our knowledge is scanty regarding allergenic proteins found in these legumes as there are few studies on allergenicity of legumes consumed in this country.

The primary objective of the study was to screen pepsin resistant proteins of different leguminous crops like, chickpea, black gram, kidney bean and Bengal gram for predicting allergenicity of these proteins. Soybean and peanut were taken as positive control where characteristic details of allergens including their molecular weights were known. In order to demonstrate allergenic potential of proteins stable to SGF digestion, specific IgE immunoblotting of soybean and chickpea CPE was performed with patient's sera allergic to soybean and chickpea.

Materials and methods

Sera of allergic patients

Five ml venous blood was collected from patients showing positive SPT and history of allergy to chickpea ($n=10$) and soybean ($n=1$) and also from normal individuals ($n=6$) who did not show history or skin reactivity to any of the allergen extracts ($n=88$) tested. Serum was separated and stored at -20°C until used. SPT and blood collection were carried out with the consent of patients and the study protocol was approved by the human ethics committee of the institute.

Chemicals

Pepsin from porcine gastric mucosa (Catalog number P 6887), purified proteins like β -amylase from sweet potato,

lipoxygenase from soybean (Type IB, lyophilized), ovalbumin from chicken egg white, lysozyme from chicken egg white, β -lactoglobulin B from bovine milk, Bovine Serum Albumin, acrylamide, N,N'-Methylene-bis-Acrylamide, sodium dodecyl sulfate, ammonium per sulfate, N,N,N',N'-Tetramethylethylenediamine, coomassie brilliant blue (R-250), wide range (205–6.5 kDa) molecular weight markers for protein, 3,3'-Diaminobenzidine tetrahydrochloride (DAB), goat anti-human IgE peroxidase conjugate were purchased from Sigma Chemical Company (St. Louis, MO, USA). Precision Plus protein dual color marker was purchased from BioRad Laboratory (Hercules, CA, USA). The anti-human IgE antibody preparation was specific for human IgE only when against purified human IgG, as evaluated by manufacturer using Ouchterlony Double Diffusion (only ODD) and immunoelectrophoresis (IEP). Purified Cry 1 Aa and Cry 1 Ab proteins were obtained as a gift from Dr. D. N. Kachru, IITR, Lucknow.

Preparation of crude protein extract (CPE)

Proteins from different leguminous crops like Soybean (*Glycine max*), Peanut (*Arachis hypogaea*), Black gram (*Phaseolus mungo*), Kidney bean (*Phaseolus vulgaris*), two varieties of Chickpea (*Cicer arietinum*) cultivars i.e., Kabuli type and desi type (also known as Bengal gram) were extracted as described by Astwood et al.⁽⁹⁾ with slight modifications. Briefly, seeds were powdered using a grinder and delipidated with n-hexane. Delipidated flour was macerated for 2 minutes in grinder along with the phosphate buffer (20 mM Na_2HPO_4 , 2 mM MKH_2PO_4 , 5.4 mM KCl, 0.5 M NaCl, pH 7.0). The mixture was agitated overnight at 4°C and centrifuged for 30 minutes at the $10,000\times g$. The supernatant was recovered, filtered through $0.45\text{ }\mu\text{m}$ syringe filter and stored in aliquots at -80°C until used. Protein content of CPE was estimated using Bovine Serum Albumin (BSA) as reference.⁽¹⁸⁾

Preparation of SGF

SGF was prepared as described by United State Pharmacopoeia.⁽¹⁹⁾ Pepsin, 3.2 mg (approximately 3460 unit activity/mg) was dissolved in 1 ml of 34 mM NaCl and 0.7% HCl, pH 1.2. Activity of each newly prepared SGF solution was defined as the production of a ΔA_{280} of 0.001 per min at pH 1.2 at 37°C measured as trichloroacetic acid (TCA) soluble products with hemoglobin as substrate. SGF solution was freshly prepared and used within the same day.

Digestibility of purified food proteins and Crude Protein Extracts in SGF and SDS-PAGE

SGF assay was performed taking purified non-allergenic proteins (β -amylase and lipoxygenase) and allergenic

proteins (ovalbumin, lysozyme and β -lactoglobulin). Cry 1 Aa and Cry1 Ab were taken to assess their stability in SGF, because *cry* genes are commonly inserted to produce transgenic crops with insect resistant property. The digestibility of all these proteins was examined as described by Roesler and Rao⁽²⁰⁾ with minor modifications.

All proteins were dissolved at a concentration of 1 mg/ml in deionized water. SGF (430 μ l) was incubated at 37°C prior to addition of 86 μ l of test protein solution. The contents of the tube were mixed by mild vortexing and the tube was immediately placed in a 37°C water bath. The reaction was stopped at different time intervals (0.25, 0.5, 1, 2, 4, 8, 15 and 60 minutes) by the addition of 60 μ l of the incubation mixture to 60 μ l of stopping solution, that is 2x Tris-tricine SDS sample buffer (containing 200 mM dithiothreitol, 4% SDS, 0.2% bromo phenol blue, 20% glycerol and 100 mM Tris, pH 8.8). SGF and protein were added directly to the stopping solution prior to the incubation for zero min control.

Each sample was boiled for 5 min at 100°C. Boiled samples (15 μ l/well) were subjected to SDS-PAGE according to the method of Laemmli,⁽²¹⁾ using 15 well, 1 mm thick, 14% polyacrylamide Tricine midi gels of 18 $\times$ 16 cm (Amersham Biosciences, San Francisco, CA, USA) at 22 mA for 4:30 h. Standard molecular weight markers were included in all gels to calculate the molecular weight of the proteins. Coomassie brilliant blue (R-250) stain was employed for all the gels. Gel images were captured using Syngene Bio Imaging System (Syngene, Cambridge, UK).

Digestibility of CPE of soybean, peanut, chickpea, black gram, kidney bean and Bengal gram was performed essentially as described above. The only difference was in starting protein concentration of various CPEs prior to SGF digestion (4 mg/ml instead of 1 mg/ml).

The resulting undigested bands were quantified using the Genetools software (Syngene, Cambridge, UK) where raw volume of the bands was recorded. The raw volume was defined as a three-dimensional size of the band where intensity was the third dimension. Each sample protein was digested at least 4 times and was subjected to reproducible SDS-PAGE and densitometry of each protein band was performed thrice. The average values have been used for preparing graphs.

Immunoblot of Soybean and Chickpea CPE

Soybean and chickpea CPE were immunoblotted with corresponding allergic patient sera. Proteins were resolved onto 12% SDS-PAGE. Resolved proteins were electrophoretically transferred onto PVDF membrane with pore size 0.45 μ m (Immobilon-P, Millipore Co., USA) according to Towbin et al.⁽²²⁾ for 2 h at 0.8 mA/cm²

in Towbin buffer with a semidry blotting unit (Amersham Biosciences, San Francisco, CA, USA). After termination of the electrophoretic transfer, membrane was blocked with 5% fat-free milk in TBS-T buffer ((20 mM Tris-HCl, pH 7.4), 500 mM NaCl containing 0.1% Tween-20) and kept for 2 hours at RT. After three washings (5 min each) with TBS-T, the strips were incubated with sera of allergic patients overnight at 4°C (dilution 1:10 in TBS-T + 1% BSA). Sera of non-sensitized populations were taken as negative control.

After 3 washes again (5 min each) with TBS-T, the membranes (incubated with chickpea and soy bean sensitized patients as well as non-sensitized patients) were further incubated for 6 h at RT with goat anti-human IgE peroxidase conjugate (Sigma Chemical Company, dilution 1:1000 in TBS-T + 1% BSA). After two additional washes (5 min each), the IgE binding proteins were visualized with 3, 3'-Diaminobenzidine tetrahydrochloride, a substrate for HRP, which yields a colored deposit. Immunoblots of soybean and chickpea CPE were matched with SGF digests of soybean and chickpea to show allergenic potential of pepsin stable proteins.

Results

Digestibility of non-allergenic and allergenic purified food proteins in SGF

Two non-allergenic food proteins, β -amylase and lipoxxygenase were completely digested within 15 seconds in the SGF. In contrast, the food allergenic proteins, lysozyme, ovalbumin and β -lactoglobulin were found to be stable up to 8, 15 and 60 min, respectively. Cry 1 Aa and Cry 1 Ab that have been inserted in several genetically modified crops (purified from *E. coli* culture) were digested completely in less than 30 seconds (Table 1, SDS-PAGE not shown).

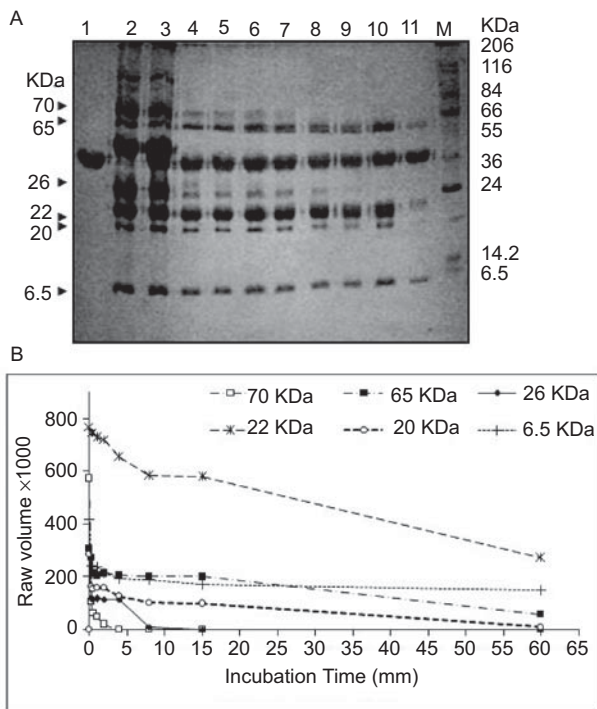
Digestibility of Crude Protein Extracts in SGF

Figure 1A shows the digestion pattern of CPE of soybean in SGF. SDS-PAGE of soybean CPE before SGF digestion (lane 2) presented 10 distinct proteins with apparent molecular weights (mol wts) ranging from 6.5 to 97 kDa. As estimated from standard mol wts marker (lane M), four proteins of approximately 65, 22, 20 and 6.5 kDa persisted up to 60 min (lane 11), while 26 and 70 kDa proteins remained stable till 8 and 2 min, respectively (lane 9 and 7). The remaining four proteins of mol wts of approximately 97, 84, 80 and 29 kDa were rapidly digested in SGF in less than 15 sec.

Densitometric analysis of the undigested proteins of soybean CPE after SGF digestion at different time intervals was performed to know relative amount and its stability

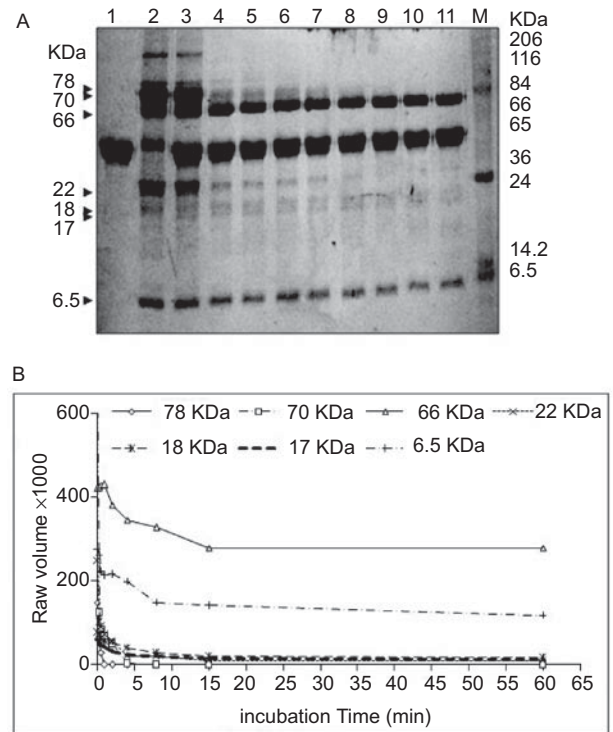
Table 1. Digestibility of purified allergenic and non-allergenic proteins in Simulated Gastric Fluid (SGF)

Protein (Purified)	SGF Digestibility time (min)
β -amylase	<0.25
Lipoxygenase	<0.25
Cry1 Aa	<0.5
Cry 1Ab	<0.25
Lysozyme	8
Ovalbumin	15
β -lactoglobulin	60

**Figure 1.** (A). SDS-PAGE of the SGF digested soybean CPE. Lane 1: Pepsin; Lane 2: soybean CPE; Lane 3: 0 min; Lane 4-11: SGF digestion pattern of soybean at time points: 0.25, 0.5, 1, 2, 4, 8, 15 and 60 min, Lane M: molecular weight marker. (B). Quantification of density of undigested soybean protein bands. Values are mean of triplicate analysis.

towards pepsin (Figure 1B). Protein of 22kDa was most abundant as shown by highest intensity. Protein of 6.5kDa remained unaffected till 60min. There was almost no change in the amount of the proteins of mol wts 65, 22 and 20kDa up to 15 min, at 60 min decrease in intensity of these was observed. The amount of the 26kDa protein was constant up to 8min and thereafter it could not be observed indicating complete digestion, while the amount of protein of 70kDa gradually decreased up to 2min and thereafter it was completely digested.

SDS-PAGE of peanut CPE showed 11 proteins ranging from 6.5 to 100kDa (Figure 2A, lane 2). Proteins of 66, 22, 18, 17 and 6.5kDa were stable to SGF digestion up to 60 min (lane 11). Proteins of 70 and 78kDa remained

**Figure 2.** (A). SDS-PAGE of the SGF digested peanut CPE. Lane 1: Pepsin; Lane 2: peanut CPE; Lane 3: 0 min; Lane 4-11: SGF digestion pattern of peanut at time points: 0.25, 0.5, 1, 2, 4, 8, 15 and 60 min, Lane M: molecular weight marker. (B). Quantification of density of the undigested peanut protein bands. Values are mean of triplicate analysis.

undigested up to 4 min and 30 sec, respectively (lane 8 and 5) following digestion. Proteins of 100, 97, 35 and 15kDa got digested in less than 15 sec in SGF. Protein of 66kDa had highest intensity followed by 6.5kDa protein as shown by densitometric analysis. The densitometry of undigested bands of peanut proteins showed that concentration of 66, 22, 18, 17 and 6.5kDa remained almost constant till 60 min while protein bands of 70 and 78kDa showed continuous decrease with increase in incubation period and degraded completely after 4 min and 30 sec, respectively (Figure 2B).

The digestion profile of black gram is shown in Figure 3A. SDS-PAGE of its CPE resulted in 13 proteins with mol wts ranging from 6.5 to 84kDa (lane 2). Protein of 47kDa was stable up to 60 min after SGF digestion (lane 11). Proteins of 30, 29, 28, 26, 24, 22 and 16kDa were stable till 15 min (lane 10). Proteins of 14 and 12kDa were digested within 2 min (lane 7). Proteins of 84, 66 and 6.5kDa was digested rapidly in less than 15 sec. Figure 3B shows densitometric analysis of remainder of undigested proteins of 47, 30, 29, 28, 26, 24, 22, 16, 14 and 12kDa of black gram. The protein of 47kDa was most abundant, followed by 30kDa and 16kDa. The amount of 47kDa protein decreased suddenly after 15 sec and was almost constant up to

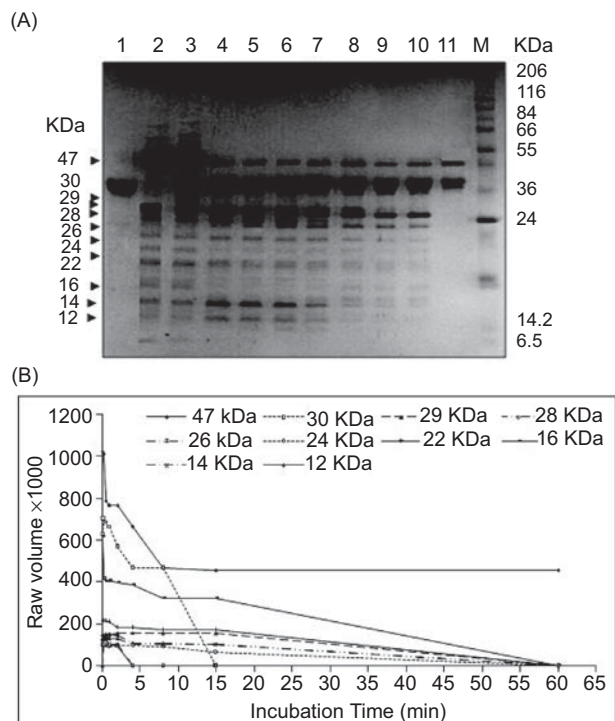


Figure 3. (A). SDS-PAGE of the SGF digested black gram CPE. Lane 1: Pepsin; Lane 2: black gram CPE; Lane 3: 0 min; Lane 4-11: SGF digestion pattern of black gram at time points: 0.25, 0.5, 1, 2, 4, 8, 15 and 60 min; Lane M: molecular weight marker. (B). Quantification of density of the undigested black gram protein bands. Values are mean of triplicate analysis.

60 min. There was a constant decrease in the amount of 30, 29, 28, 26, 24, 22 and 16 kDa proteins with time and they were fully digested by 15 min. The amounts of 14 and 12 kDa proteins decreased with time up to 2 min, after that both were completely digested as these could not be seen after staining.

Figure 4A shows digestion profile of kidney bean. SDS-PAGE of kidney bean CPE showed 8 proteins with mol wt ranging from 6.5–116 kDa (lane 2). Protein bands of 45, 29, 24, 20 and 6.5 kDa were stable up to 1 h after SGF digestion (lane 11). Remaining of the three proteins of 116, 84 and 27 kDa were digested in less than 15 seconds. Protein of 45 kDa was most abundant, followed by 29 kDa. There was decrease in the amount of all the five stable proteins of 45, 29, 24, 20 and 6.5 kDa at 60 min in comparison to the earlier time points (Figure 4B).

SDS-PAGE of Bengal gram CPE showed eight proteins with mol wt ranging from 20 to 97 kDa (Figure 5A, lane 2). Out of eight, only one protein of approximately 20 kDa remained stable till 2 minutes, as shown by SGF digestion and densitometric analysis (Figure 5A, lane 7 and Figure 5B). Remaining seven proteins were digested in less than 15 seconds.

SDS-PAGE of chickpea CPE showed 10 proteins with mol wts ranging from 18 to 84 kDa (Figure 6A, lane 2). Five proteins of 55, 45, 35, 20 and 18 kDa remained

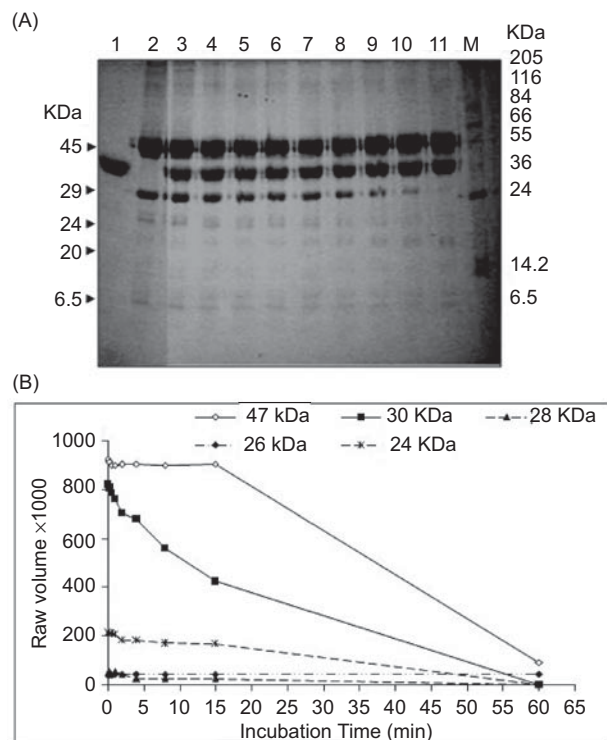


Figure 4. (A). SDS-PAGE of the SGF digested kidney bean CPE. Lane 1: Pepsin; Lane 2: kidney bean CPE; Lane 3: 0 min; Lane 4-11: SGF digestion pattern of kidney bean at time points: 0.25, 0.5, 1, 2, 4, 8, 15 and 60 min; Lane M: molecular weight marker. (B). Quantification of density of the undigested kidney bean protein bands. Values are mean of triplicate analysis.

undigested till 60 min in SGF (lane 11). Proteins of 70 kDa and 64 kDa degraded completely after 15 min (lane 10). A new protein band of approximately 42 kDa appeared after 15 sec of digestion (lane 4) and was stable up to 60 min (lane 11). This 42 kDa protein was not present in the CPE of chickpea and may be the hydrolyzed product of some larger protein. Proteins of 84, 38 and 29 kDa were easily digested in SGF in less than 15 sec. Figure 6B shows the time course of digestion proteins determined by densitometry of 70, 64, 55, 45, 42, 35, 20 and 18 kDa proteins of chickpea. The protein of 35 kDa was most abundant and amount of 35 and 20 kDa proteins remained almost constant up to 60 min while that of 70, 64, 55 and 18 kDa constantly decreased with time. Intensity of 42 kDa protein increased with time up to 60 min.

IgE Immunoreactivity of Soybean and Chickpea CPE

In order to demonstrate allergenic potential of undigested proteins, IgE reactivity of soybean and chickpea CPE was analyzed by immunoblotting with sera of soybean and chickpea hypersensitive patients. Immunoblots with individual and pooled sera of chickpea allergic

patients demonstrated seven IgE binding proteins of 70, 64, 55, 45, 35, 20 and 18 kDa (Figure 7, lane 6–10) and proteins of similar mol wts were stable up to 2 min in SGF (Figure 7, lane 5). Immunoblot of soybean CPE showed five IgE binding proteins of 70, 65, 26, 22 and 20 kDa mol wts (Figure 8, lane 6), that were similar to mol wts of proteins stable in SGF up to 2 min (Figure 8, lane

5). Immunoblot of soybean and chickpea CPE with TBS and normal human sera did not show binding to any of the proteins. Comparative SDS-PAGE protein profile of soybean and chickpea CPE with their pepsin digests at 2 min depicts easily digestible (stainable for < 1 min) and stable proteins. IgE immunoblots of CPE without SGF digestion when compared to 2 min pepsin digests clearly

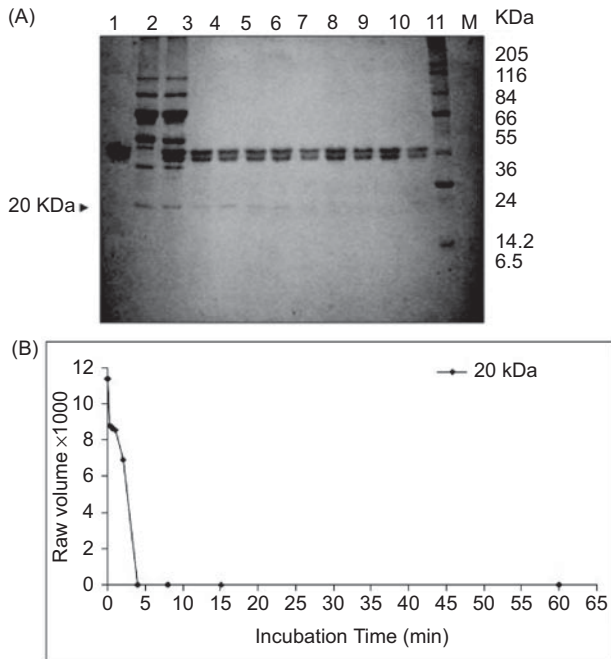


Figure 5. (A). SDS-PAGE of the SGF digested Bengal gram CPE. Lane 1: Pepsin; Lane 2: Bengal gram CPE; Lane 3: 0 min; Lane 4–11: SGF digestion pattern of Bengal gram at time points: 0.25, 0.5, 1, 2, 4, 8, 15 and 60 min; Lane M: molecular weight marker. (B). Quantification of density of the undigested Bengal gram protein band. Values are mean of triplicate analysis.

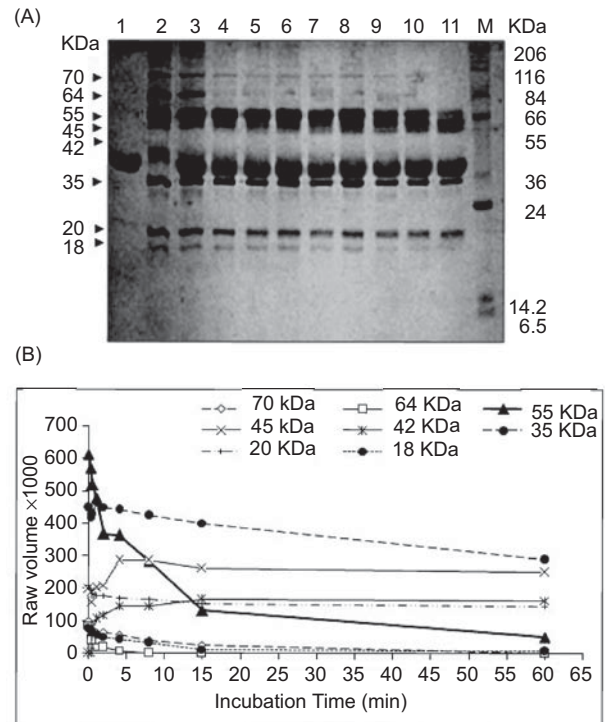


Figure 6. (A). SDS-PAGE of the SGF digested chickpea CPE. Lane 1: Pepsin; Lane 2: chickpea CPE; Lane 3: 0 min; Lane 4–11: SGF digestion pattern of chickpea at time points: 0.25, 0.5, 1, 2, 4, 8, 15 and 60 min; Lane M: molecular weight marker. (B). Quantification of density of the undigested chickpea protein bands. Values are mean of triplicate analysis.

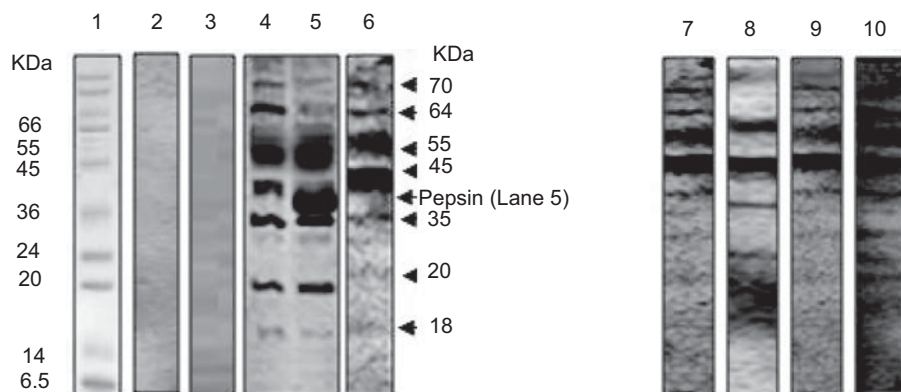


Figure 7. Immunoblot of chickpea-sensitive patient sera showing IgE binding with pepsin resistant proteins of chickpea. Lane 1: molecular weight markers; Lane 2: chickpea CPE incubated with pooled normal human sera and antihuman IgE-HRP linked; Lane 3: chickpea cPE incubated with PBS and antihuman IgE-HRP linked; Lane 4: SDS-PAGE of chickpea CPE; Lane 5: SGF digestion pattern of chickpea at 2 minutes; Lane 6: Immunoblot of chickpea CPE with pooled sera of chickpea sensitive patient; Lane 7–10: Immunoblot of chickpea CPE with individual serum of chickpea sensitive patient.

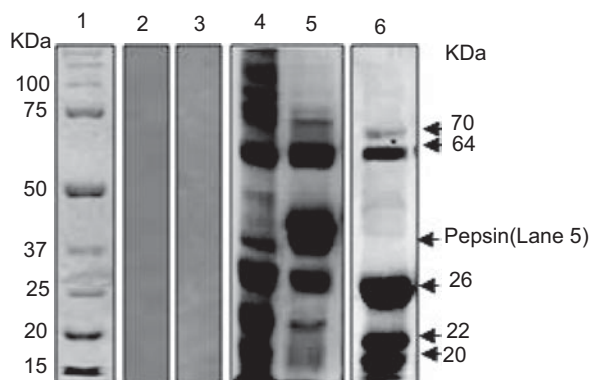


Figure 8. Immunoblot of soybean-sensitive patient sera showing IgE binding with pepsin resistant proteins of soybean. Lane 1: molecular weight markers; Lane 2: soybean CPE incubated with pooled normal human sera and antihuman IgE-HRP linked; Lane 3: soybean CPE incubated with PBS and antihuman IgE-HRP linked; Lane 4: SDS-PAGE of soybean CPE; Lane 5: SGF digestion pattern of soybean at 2 minutes; Lane 6: IgE Immunoblot of soybean CPE with soybean sensitive patient Sera.

demonstrate that pepsin sensitive proteins did not show any IgE binding, whereas pepsin stable proteins showed IgE binding.

Discussion

Stability to digestion against gastric enzymes is thought to be an important feature of allergenic food protein.^(1-3,23) Stable proteins may be important for both sensitization of the immune system after reaching intestinal mucosa and for the elicitation of gastrointestinal and other symptoms of food allergy.^(1-3,8) This assumption was validated in this laboratory using known allergenic and non-allergenic purified food proteins. Our results with purified lysozyme, ovalbumin and β -lactoglobulin (all allergenic proteins) showed stability in SGF for more than 8 minutes while β -amylase and lipoxigenase (non-allergenic proteins) were digested within 15 seconds, which are in agreement with the earlier studies.^(9,24) SGF digestion of chestnut and cherry CPE has been used successfully by Lee et al.⁽⁶⁾ and Scheurer et al.⁽⁷⁾ for allergenicity determination of proteins.

Although soybean, peanut, egg, milk, wheat, fish, crustacean and tree nuts are considered to be the most common food allergens worldwide,⁽¹⁾ the list of the most allergic products differs from country to country according to the eating habits and traditions of the people. Leguminous crops are extensively consumed in India, Thailand, Sri Lanka, and Indonesia and provide major source of affordable proteins, therefore, it was not surprising to find a large population sensitized to legumes in India.⁽²⁵⁾ Black gram induces IgE mediated reactions in asthma and allergic rhinitis patients.⁽²⁶⁾ Patil et al.⁽²⁷⁾ reported chickpea to be an important source of allergens

that caused IgE mediated hypersensitivity reactions ranging from rhinitis to anaphylaxis.

Studies carried out so far in allergic population mainly focused on a particular legume like chickpea/lentil and its allergens; however exposure to other legumes that may cause pathogenic/allergenic symptoms, were not taken into consideration. Literature on allergenic proteins of tropical legumes and their properties is scarce, except Soybean and Peanut. Some data exist on adverse reaction to temperate legumes including pea and lentil.⁽²⁸⁾ Chickpea allergy is reported in Spain where it is commonly consumed⁽²⁹⁾ and lentil allergy is more common in the Mediterranean area.⁽¹⁵⁾ Therefore, the present study was aimed to identify non-digestible and perhaps allergenic proteins of different leguminous crops commonly consumed in India using SGF assay as a predictive tool for allergenicity.

In vitro digestibility of CPE of two food crops like soybean and peanut whose allergens are known and well characterized^(30,31) was performed. Chickpea and black gram CPE whose allergens are not well characterized were investigated for digestibility. The SGF digestibility of kidney bean and Bengal gram CPE was performed to identify non-digestible proteins, which may probably be allergenic.

Four proteins of 65, 22, 20 and 6.5 kDa of soybean CPE remained undigested in SGF till 60 min. On the basis of mol wts, it can be speculated that these proteins probably correspond to soybean major allergen alpha subunit of Gly mBd 60 K allergen,⁽³²⁾ G2 glycinin,⁽³⁰⁾ soybean minor allergen Kunitz-trypsin inhibitor,⁽³³⁾ and Gly m2 allergens,⁽³⁴⁾ respectively. The 26 kDa protein was stable up to 8 min and may possibly be Gly m Bd 28 K.⁽³⁵⁾ Like soybean, mol wts of peanut proteins found stable in SGF matched to mol wts of proteins known to be allergenic.

There was a correlation between stability and allergenicity as peanut CPE electrophoresis post-SGF digestion resulted in 66, 18, 17 and 7 kDa bands (stable up to 60 min) that have similar mol wt to major allergen Ara h1,⁽³⁶⁾ oleosin monomer,⁽³⁷⁾ Ara h2,⁽²⁾ and tyrosin and chymotrypsin inhibitor,⁽³⁸⁾ respectively. In black gram protein of 47 kDa which was stable up to 60 min and proteins of 29, 28, 26, 24, 22, 16, 14 and 12 kDa were stable from 2 to 15 min in SGF may be allergenic as most of them have similar mol wt to IgE binding proteins of black gram reported by Kumari et al.⁽²⁶⁾ In kidney bean, 50, 29, 24, 20 and 6.5 kDa proteins and in Bengal gram, 20 kDa were found to be stable after SGF digestion. Chickpea CPE SGF digestion resulted in seven non-digestible proteins of 70, 64, 55, 45, 35, 20 and 18 kDa. Proteins of similar mol wts have been shown to be allergenic by other investigators using immunoblot^(27,39) and thus substantiate the hypothesis that non-digestible proteins may be allergenic.

In order to confirm that undigested proteins are having allergenic properties, proteins of soybean and chickpea CPE were immunoblotted with sera from patients allergic to these crops. In the case of soybean CPE 70, 65, 26, 22 and 20 kDa proteins were found to have IgE binding potential. Proteins of similar mol wts were stable in SGF. Interestingly, soybean proteins that got digested earlier than 30 seconds in SGF did not show any IgE binding, corroborated the hypothesis that stability to SGF digestion may be related to allergenicity.

Immunoblotting of SDS-PAGE separated chickpea proteins with sera of individual as well as pooled patients demonstrated seven IgE binding proteins of 70, 64, 55, 45, 35, 20 and 18 kDa as reported in our previous study.⁽²⁵⁾ Mol wts of five proteins (70, 64, 45, 35 and 18 kDa) out of seven were similar to the finding of Patil et al.⁽²⁷⁾ and Niphadkar et al.⁽³⁹⁾ Reports of Patil et al.⁽²⁷⁾ regarding allergenic proteins ranging from 20 to 70 kDa (70, 64, 35 and 26 kDa) and Niphadkar et al.⁽³⁹⁾ of 10 to 70 kDa (66, 45, 34.7, 24, 18.4 and 14.3) in chickpea are very similar to our findings.

However, further research is needed for 55 and 20 kDa proteins as these proteins showed IgE binding as well as remained undigested in SGF. Ireneo et al.⁽⁴⁰⁾ have detected multiple IgE binding proteins/peptides in both crude as well as boiled chickpea extract, in the mol wt range of 10–106 kDa, of which the majority were heat stable allergens. These could be responsible for clinical sensitivity to chickpea. Therefore, immunoblot results of our study reiterate that proteins that are resistant to pepsin digestion may have allergenic potential as well.

In this article, we are reporting for the first time non-digestible proteins of different legume crops remaining after SGF digestion. It was found that seven proteins from chickpea (70, 64, 55, 45, 35, 20 and 18 kDa), ten from black gram (47, 30, 29, 28, 26, 24, 22, 16, 14 and 12 kDa), five from kidney bean (45, 29, 24, 20 and 6.5 kDa) and one protein of 20 kDa from Bengal gram were stable after SGF digestion. All these proteins from different crops may have allergenic potential and needs to be tested for their allergenicity using sera of patients having allergy to these crops. We are demonstrating for the first time that non-digestible proteins of soybean and chickpea were showing allergenicity as revealed by their IgE binding properties. Similarity of mol wts of undigested proteins of soybean, peanut, chickpea and black gram to their reported allergens along with demonstration of IgE binding properties of undigested soybean and chickpea proteins depict utility of simple SGF digestion in initiation of evaluating allergenicity of a legume protein.

Further studies are required to characterize these non-digestible proteins of different leguminous crops so that it may find application in the prevention and

treatment of allergic reactions in sensitized individuals. This study may also help in the development of hypo allergenic genetically modified legumes, if the protein found allergenic could be deleted by genetic modification without losing legumes major characteristics.

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