



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

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ABSTRACT

Legumes are the major elicitors of IgE-mediated food allergy in many countries of the world. Purified major allergens are prerequisite for component resolved diagnosis of allergy. The present study was aimed to isolate and characterize a major allergenic protein from blackgram (*Phaseolus mungo*). Respiratory allergy patients with history of blackgram allergy were skin prick tested (SPT) and sera were collected from SPT positive patients. The blackgram extract was fractionated using a combination of anion exchange and hydrophobic interaction chromatography. The purified protein was characterized by indirect ELISA, immunoblot, ELISA inhibition, SPTs, stripped basophil histamine release, lymphoproliferation assay and digestibility assay. The purified protein separated at 28 kDa on 12% gel and showed IgE binding with 81% of blackgram hypersensitive patients' sera on immunoblot indicating it to be a major allergen. Periodic Acid Schiff's and meta-periodate treatment staining detected it to be a glycoprotein. The 28 kDa protein recognized 7/9 (77.8%) of blackgram positive patients by SPT, where as all 9 patients showed significant histamine release on stimulation with protein as compared to controls. The 28 kDa protein remained stable up to 15 min on incubation with SGF. Bands of 14–16 kDa appeared after 15 min of pepsin digestion that remained stable up to 60 min of incubation. However, purified protein degraded within 5 min after incubation with SIF. The N-terminus-12 residues sequence of 28 kDa protein was GRREDDYDNLQL. A stretch of residues 'DDYDNLQL' showed homology with Rho-specific inhibitor of transcription termination ($E=0.42$, Identity=87%) and NBS-LRR type disease resistant protein from peanut (*Arachis hypogaea*) ($E=2$, Identity=77%). In conclusion, the purified 28 kDa protein is a potent major allergen that may have implication in diagnosis of blackgram allergy.

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Introduction

Legumes such as peanut, soybean, lentil and chickpea have been recognized as important triggers of food allergy in the US, Europe and Asian countries (Ibanez Sandin et al. 1999; Lin et al.

2006; Maleki et al. 2010; Patil et al. 2001). Ingestion or inhalation of vapours during cooking of legumes had provoked diarrhea, angioedema, or severe anaphylactic reactions in predisposed individuals (Burks et al. 1999; Kalogeromitros et al. 1996; Martin et al. 1992; Niphadkar et al. 1997; Sicherer et al. 2000; Wassenberg and Hofer 2007). Blackgram (*Phaseolus mungo*), a protein rich legume is extensively used for preparing many delicious dishes worldwide and is identified as a major food allergen in asthma and rhinitis patients (Kumari et al. 2006).

Many food allergens from legume family have been isolated and characterized in the past decades. These include Ara h 1–Ara h 8 from peanut, Len c 1 and Len c 2 from lentil and Gly m 1–Gly m 4 from soybean and Pis s 1 and Pis s 2 from pea (Breiteneder and Ebner 2000; Burks et al. 1991, 1992, 1998; Lopez-Torres et al. 2003; Mittag et al. 2004a). The availability of purified allergen components, makes it possible to delineate the patient's individual IgE reactivity profile and to identify the disease-causing allergen molecules i.e. component-resolved diagnosis (CRD) (Heiss et al.

Abbreviations: SPT, skin prick test; CRD, component-resolved diagnosis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue; NCM, nitrocellulose membrane; PBMCs, peripheral blood mononuclear cells; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; BSA, bovine serum albumin; PAS, Periodic Acid Schiff's; PVDF, polyvinylidene difluoride membrane.

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1999; Hage-Hamsten et al. 2002). Purified allergens can also be used to supplement the allergen-extract with the relevant allergens that are under-represented (spiking of extracts), thereby enhancing the sensitivity of the extract (Ballmer-Weber et al. 2007). Soybean extract spiked by purified recombinant Gly m 4 showed higher diagnostic sensitivity than that of native extract-based test (Mittag et al. 2004b).

Previously, IgE mediated food allergy to blackgram was established by double blind placebo controlled food challenge test in asthma and rhinitis patients (Kumari et al. 2006). Immunoblotting with hypersensitive patients' sera detected 14 IgE-binding components with 8 major allergens of 78, 56, 47, 43, 40, 30, 28 and 16 kDa in blackgram extract. The present study was aimed to isolate and characterize a major allergenic protein of blackgram using column chromatography and standard immunobiochemical methods.

Methods

Preparation of extracts

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

Study subjects

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

Purification of an allergenic protein from blackgram

The whole seed black gram extract was subjected to an ion exchange chromatography using fast protein liquid chromatography. Lyophilized protein (50 mg) was reconstituted in 20 mM Tris buffer (pH 7.9) and loaded onto 5 ml Q-Sepharose gel column equilibrated with same buffer. After washing unbound protein in equilibration buffer, the bound proteins were eluted with a linear salt gradient of 0–600 mM NaCl. All the fractions of each resolved peak were pooled, dialyzed, lyophilized and run on SDS-PAGE. The fractions were tested for IgE-binding on immunoblot using pooled patients' sera. Fractions containing a distinct band of 28 kDa and two faint bands of 47- and 16 kDa were subjected further to hydrophobic interaction chromatography. The 28 kDa enriched fractions were dialyzed and equilibrated with Tris buffer (20 mM, pH 7.9) containing 1 M ammonium sulfate. This was loaded onto Phenyl Sepharose column equilibrated with 20 mM Tris buffer, pH 7.9 containing 1 M ammonium sulfate. The unbound proteins were collected by washing the column with the same equilibration buffer. Bound proteins were eluted with 20 mM Tris buffer, pH 7.9. The unbound and eluted fractions were run on SDS-PAGE. The unbound fraction containing 28 kDa protein was collected, dialyzed against PBS, filtered sterilized and finally lyophilized and reconstituted to protein concentration of 10 mg/ml.

Specific IgE

Specific IgE binding against purified protein/whole seed blackgram extract were determined by indirect ELISA (Kumari et al. 2006). Briefly, 200 ng of purified protein and/or 1 µg of blackgram extract in 100 µl of carbonate-bicarbonate buffer (pH 9.6) was coated per well, overnight at 4 °C (Nunc maxisorp plates, Nalge Nunc International, Roskilde, Denmark). After washing with PBS-Tween 20 (0.05%), non specific sites were blocked with 3% defatted milk for 3 h at 37 °C. The plates were washed and incubated with 1:10 (v/v) sera of blackgram hypersensitive patients' at 4 °C. Serum from healthy volunteers were used as negative control. The plates were washed and incubated with anti-human IgE-horse radish peroxidase 1:1000 (v/v) (Sigma) for 3 h at 37 °C. Color was developed with orthophenylene diamine. The reaction was stopped after 20 min by adding 5 N H₂SO₄ and the absorbance was read at 492 nm.

ELISA inhibition

The allergenic potency of purified 28 kDa blackgram protein was determined by ELISA inhibition. Inhibition of IgE binding was assessed with serum pools of blackgram positive patients' preincubated with 0.1, 1, 10, 100, 1000 and 10,000 ng of 28 kDa purified protein and raw blackgram extract as inhibitors. The mixture was added to the solid phase bound raw blackgram extract in ELISA plate. Here blackgram positive patients' pooled sera without inhibitor was taken as a positive control. ELISA inhibition was also carried out using *Curvularia lunata* extract as an inhibitor. Percent-age inhibition was calculated as described below:

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

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

Black gram extract (80 µg per lane) and purified protein (20 µg per lane) was loaded on a 12% reducing gel following procedure described by Laemmli (1970). The proteins were stained with Coomassie brilliant blue (CBB) or silver stained.

IgE immunoblot

SDS-PAGE resolved purified protein/blackgram extract was transferred on to nitrocellulose membrane (NCM) as described earlier (Towbin et al. 1979). The unbound sites were blocked with 3% defatted milk for 3 h at 37 °C. The NCM strips were washed and incubated with 1:10 (v/v) blackgram hypersensitive patients' sera in PBS, overnight at 4 °C. A pool of normal human sera ($n=3$) was used as negative control. The strips were washed and incubated with 1:1000 (v/v) anti-human IgE peroxidase (Sigma). The IgE binding was detected by adding 3,3-diaminobenzidine with hydrogen peroxidase in sodium acetate buffer (pH-5.0)

Skin test with blackgram extract and purified protein

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

skin reactions. Blood was collected from patients ($n=9$) showing marked positive reaction to blackgram extract and purified protein.

Stripped basophil histamine release assay

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

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

Lymphocyte proliferation and cytokine analysis on allergen challenge

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of blackgram hypersensitive patients' ($n=9$) by Ficoll Hypaque centrifugation (Sigma Chemical Co.). Cells were washed 3 times with PBS and resuspended in RPMI-1640 supplemented with 23 mM sodium bicarbonate, 25 mM HEPES, non-essential amino acids and vitamins, streptomycin (100 U/ml), penicillin (100 $\mu\text{g}/\text{ml}$) and 10% fetal calf serum. Cell viability was determined by adding 20 μl of trypan blue to 20 μl of cells and the viable numbers of cells were adjusted to 10^6 cells/ml. For lymphoproliferation assay, 100 μl of cells (10^5 cells/well) were cultured in RPMI media and challenged with 10 μg of blackgram extract or purified protein. Phytohaemagglutinin (10 μg) was used as positive control and no stimulant was added to negative control. The culture plates were incubated for 72 h and supernatant collected for cytokine assay. Cells were washed with RPMI and incubated for 2 h with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 100 $\mu\text{g}/\text{ml}$). The cells were lysed in acidic isopropanol solution and the absorbance read at 570 nm. OD values more than or equal to three times of the negative control were considered as positive stimulation index.

IL-4 and IFN- γ levels in culture supernatants were estimated by ELISA following manufacturer's protocol (BD Pharmingen OptEIA kits).

Digestibility of protein

The digestibility of purified protein and blackgram was examined in simulated gastric fluid (SGF), as described by [Astwood et al. \(1996\)](#). SGF was prepared as described in the United States Pharmacopoeia ([Singh et al. 2006](#); [Sharma et al. 2011](#)) containing 0.03 M NaCl, 0.32 (w/v) percentage of pepsin A (Sigma Chemical Co.), pH 1.2. Briefly, 28 kDa purified protein (680 μg) was treated separately with 200 μL of prewarmed SGF. Digestion was proceeded at 37°C with continuous shaking, and an aliquot (20 μL) of this digest was periodically withdrawn (at 0.5, 1, 5, 15, 30, 45, and 60 min) for 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 6.0 μL of Na_2CO_3 solution (200 mM/L). The mixture was then boiled for 5 min and stored at -20°C until further analyses. As control, each protein sample was treated with SGF that did not contain pepsin A and then processed as described above.

The purified protein was also digested in simulated intestinal fluid (SIF). SIF was prepared as described in the United States Pharmacopoeia ([Singh et al. 2006](#)) containing 10 mg/ml of pancreatin (Sigma Chemical Co.) in 0.05 M KH_2PO_4 , pH 7.5. Purified protein was

digested in SIF by incubation at 37°C for 0.25, 1, 4, 8, 16 and 24 h. The ratio of pancreatin to test protein was about 13:1 (w/w). The reaction was immediately stopped by placing the tubes in boiling water bath for 10 min. The end products were visualized by SDS-PAGE ([Laemmli 1970](#)).

Protease activity

To determine the protease activity, 50 μg purified protein and blackgram extract (5 $\mu\text{g}/\mu\text{l}$ in PBS) were incubated overnight at 37°C in the wells perforated in 0.1% agarose gel containing 50 μg of bovine serum albumin (BSA), casein hydrolysate or gelatin individually. A serine protease (Epi p 1) from mold *Epicoccum purpurascens* was used as a positive control and PBS was used as a negative control. The plates were stained with CBB. The lightly stained area around the wells indicates the proteolytic activity of protein.

Periodic Acid Schiff's (PAS) staining

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

Effect of periodate oxidation on IgE binding

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

N-terminal amino acid sequencing

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

Statistical analysis

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

Results

Purification of 28 kDa protein from blackgram extract

The blackgram extract was fractionated into five major peaks on Q-sepharose gel ([Fig. 1a](#)). All fractions of each resolved peak were pooled, dialyzed, lyophilized and run on SDS-PAGE. The protein profile of unbound and eluted protein fractions is presented in [Fig. 1b](#). Peak 1 showed a distinct band of 28 kDa and two faint bands of 47 and 16 kDa. In order to purify the proteins further, fractions of

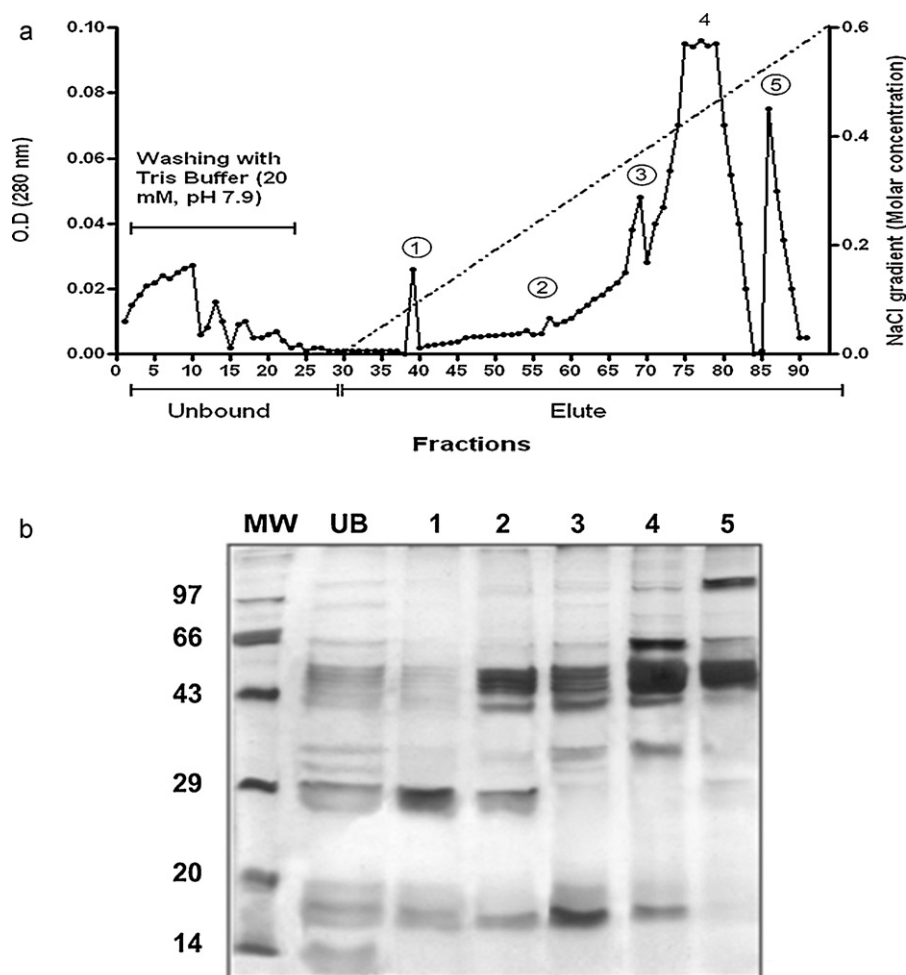


Fig. 1. (a) Elution profile of blackgram proteins on Q-sepharose anion exchange column. Unbound proteins were eluted with 60 ml of Tris buffer (20 mM, pH 7.9) at a flow rate of 2 ml/min. Bound proteins were eluted with a salt gradient from 0 to 0.6 M sodium chloride in Tris buffer (20 mM, pH 7.9) at 1 ml/min. OD at 280 nm (Left-Y-axis); NaCl gradient (0–0.6 M) (Right Y-axis). (b) Protein profile of anion exchange eluted fractions. MW, molecular weight marker; UB, unbound fraction; lane 1, fractions of peak 1; lane 2, pool of peak 2; lane 3, pool of peak 3; lane 4, pool of peak 4; lane 5, pool of peak 5. The protein bands were visualized by silver staining.

peak 1 were loaded onto phenyl Sepharose column. The 28 kDa protein appeared in the unbound fraction as a single band on SDS-PAGE and was recognized by blackgram hypersensitive pooled patients' sera on immunoblot (Fig. 2a and b). The yield of this 28 kDa protein was 1.12 mg/g of blackgram protein.

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

Immunoassays

Specific IgE binding of purified protein (28 kDa), and blackgram extract was determined by ELISA using 22 blackgram positive patients' sera. All 22 sera samples showed elevated IgE levels (ODs 0.39–0.92) against blackgram extract whereas 18/22 of sera showed high IgE titer (ODs 0.29–0.63) against purified protein (Table 1). Specific IgE values ≥ 3 times of control (blackgram extract OD 0.130, purified protein OD 0.088) were considered positive cut off for ELISA. There was a significant correlation of specific IgE reactivity of purified protein and blackgram extract ($r=0.5626$, $P=0.0064$).

IgE binding of 28 kDa protein was further assessed by immunoblotting with individual patients' sera ($n=22$) having elevated specific IgE levels to blackgram. Of these ($n=22$), 18 (81.8%)

sera showed IgE binding with purified protein demonstrating it to be a major allergen (Fig. 3a). Four patients' sera that did not show IgE binding to this protein may be sensitized to other protein fractions of blackgram.

Biopotency of 28 kDa protein

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

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

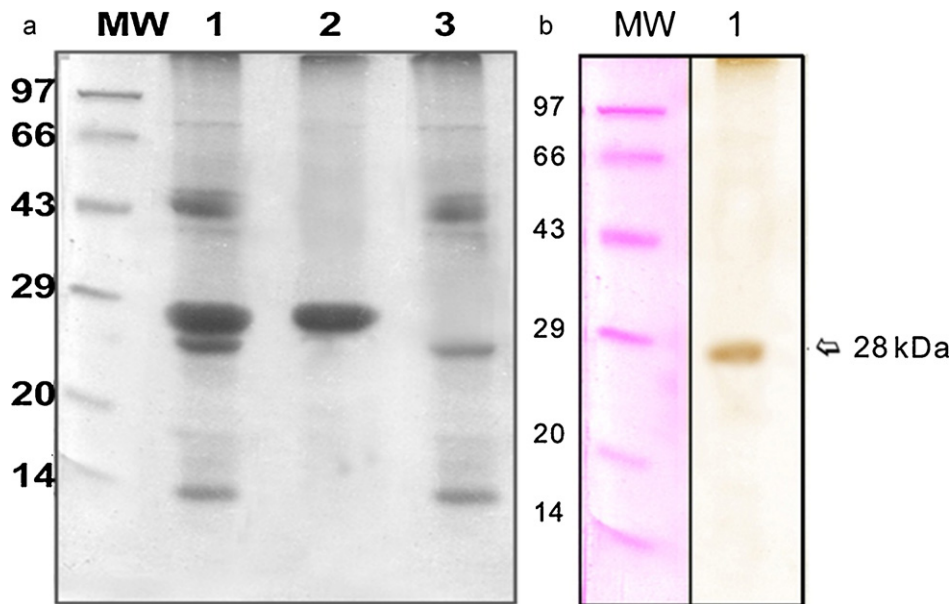


Fig. 2. (a) Protein profile of eluted fractions from phenyl sepharose hydrophobic interaction column. MW, molecular weight marker; lane 1, pool 1 from 1 step (Q-Sepharose chromatography), lane 2, phenyl sepharose unbound; lane 3, fraction eluted with Tris-HCl (20 mM, pH 7.9). The protein bands were visualized by staining with CBB. (b) Immunoblot of purified protein using blackgram hypersensitive pooled patients' sera. MW, molecular weight marker, lane 1: purified protein.

ELISA inhibition

The specificity and the allergenic potency of purified 28 kDa protein were assessed by ELISA inhibition using blackgram hypersensitive pooled patients' sera. A dose dependent inhibition of IgE binding to solid phase blackgram extract was observed, when serum pool was incubated with increasing concentrations (0.1–10,000 ng) of both crude extract and purified protein, separately (Fig. 3b). Approximately 142 ng of purified protein resulted in 50% inhibition of IgE binding to blackgram extract (solid phase), whereas 50% inhibition of IgE binding to solid phase blackgram was achieved by 14 ng of blackgram extract (self extra extract) used as

inhibitor. The purified protein was able to inhibit 61% of the IgE binding to solid-phase blackgram extract in its highest amount used (10 μ g) as inhibitor. Less than 10% inhibition of IgE binding to blackgram extract was observed when pooled sera was preincubated with *Curvularia lunata*, extract.

Table 1

Specific IgE values against blackgram extract and purified 28 kDa protein with blackgram hypersensitive patients' sera by ELISA.

Patients no.	Blackgram extract (OD)	Purified 28 kDa protein (OD)
1	0.41	0.20
2	0.39	0.33
3	0.92	0.33
4	0.52	0.29
5	0.66	0.32
6	0.71	0.51
7	0.69	0.39
8	0.48	0.38
9	0.66	0.42
10	0.59	0.31
11	0.67	0.33
12	0.42	0.18
13	0.50	0.17
14	0.40	0.13
15	0.66	0.37
16	0.91	0.63
17	0.45	0.43
18	0.47	0.52
19	0.68	0.47
20	0.63	0.62
21	0.42	0.30
22	0.52	0.35
C1	0.13	0.08
C2	0.13	0.09
C3	0.13	0.09

Correlation coefficient $r=0.5626$, $P=0.0064$.

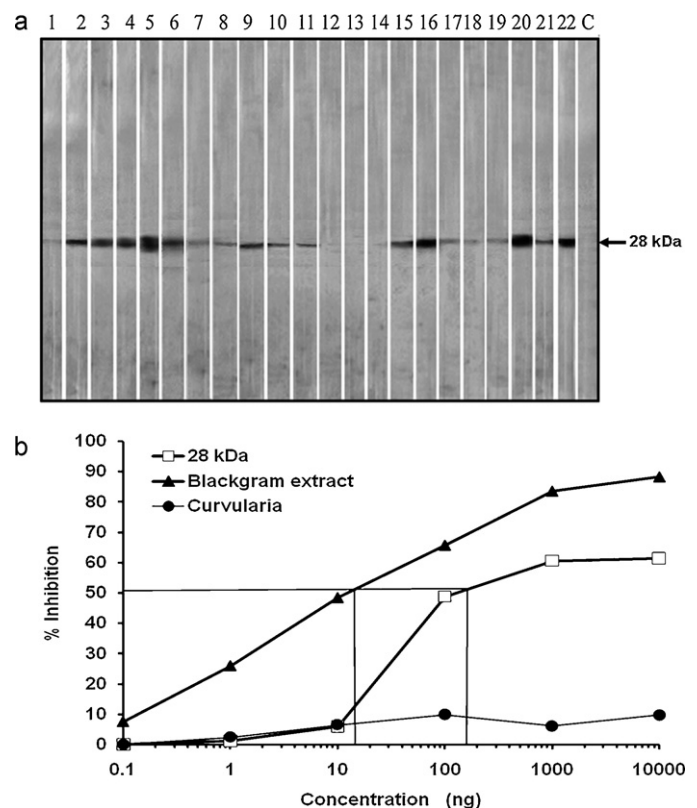


Fig. 3. (a) IgE binding of purified 28 kDa protein with individual patients' sera (1–22) on immunoblot. C: purified protein probed with control sera. (b) IgE ELISA inhibition of blackgram extract (solid phase) extract using blackgram hypersensitive pooled patients' sera incubated with 0.1–10,000 ng of blackgram extract, purified protein (28 kDa) and *Curvularia lunata* extract.

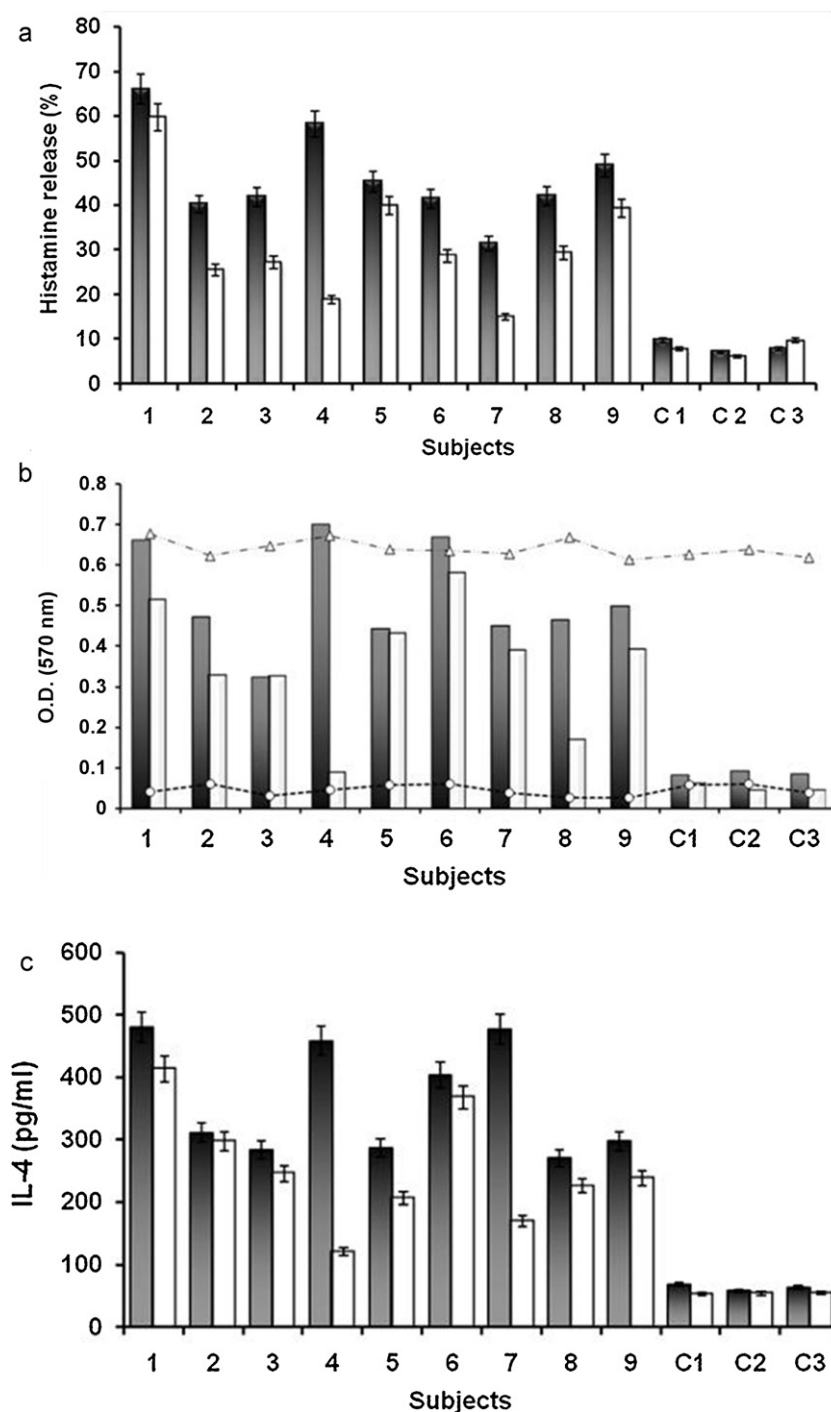


Fig. 4. (a) Histamine released from stripped basophils re-sensitized with individual patients' sera ($n=9$) on challenge with blackgram extract (■) and 28 kDa purified protein (□), separately. C1–C3: controls. (b) Lymphoproliferation assay. PBMCs from blackgram hypersensitive patients demonstrated significant proliferation when challenged with 5 μ g of blackgram (■) extract or purified 28 kDa protein (□), whereas PBMCs from nonallergic controls (C1–C3) did not show significant proliferation. PBMCs stimulated with 5 μ g phytohemagglutinin (△) and without stimulation were taken as positive and negative controls (○). (c) T_H2 cytokine IL-4 titer in the culture supernatant on stimulation with blackgram extract (■) and 28 kDa purified protein (□). C1–C3: controls.

Stripped basophil histamine release

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

histamine release with purified protein and blackgram extract ($r=0.67$, $P=0.048$).

Lymphoproliferation assay

Lymphocyte proliferation was investigated in blackgram sensitized patients' PBMCs by stimulation with 5 μ g of purified protein or blackgram extract. Purified 28 kDa protein induced significant

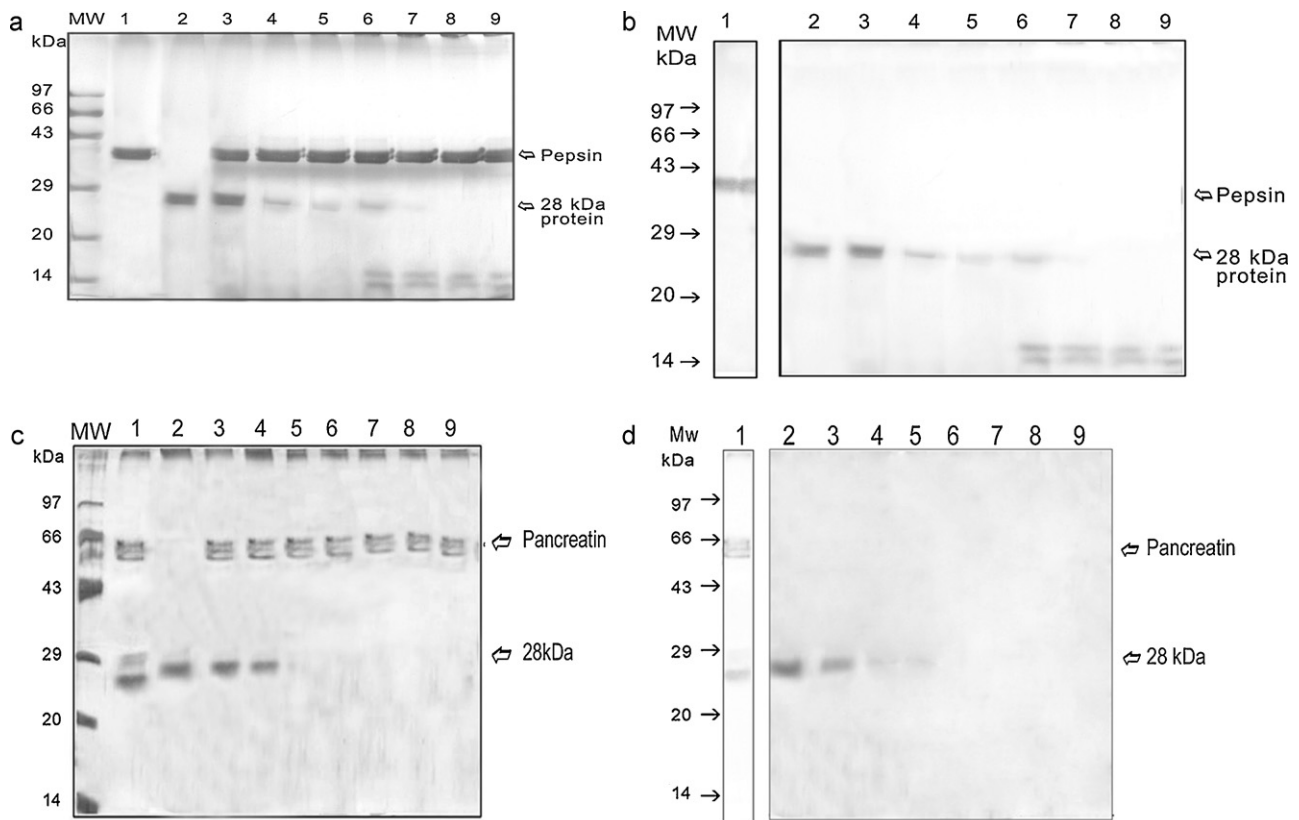


Fig. 5. (a) Simulated gastric fluid (SGF) digestion of 28 kDa protein. MW, molecular weight marker; lane 1, pepsin; lane 2, 28 kDa protein (undigested); lanes 3–9, 28 kDa. Protein (34 μ g) was incubated in SGF for 0.5, 1, 5, 10, 15, 30 and 60 min. The protein (digest) was electrophoresed on SDS-PAGE and visualized by silver staining. (b) Western blot analysis of 28 kDa protein digested in SGF with serum pool of blackgram hypersensitive patients. Lane 1, pepsin (ponceau stained); lane 2, 28 kDa protein; lanes 3–9, purified protein incubated with SGF for 0.5, 1, 5, 10, 15, 30 and 60 min. (c) Simulated intestinal fluid (SIF) degradation of 28 kDa protein of blackgram. After electrophoresis, the protein (digest) was visualized by silver staining. Mw, molecular weight marker; lane 1, pancreatin; lane 2, 28 kDa protein (untreated); lanes 3–9, purified protein incubated with SIF for 0.5, 1, 5, 10, 15, 30 and 60 min. (d) Western blot analysis of 28 kDa protein of blackgram digested with SIF with serum pool of blackgram hypersensitive patients. Lane 1, pancreatin; lane 2, 28 kDa protein (untreated); lanes 3–9, purified protein incubated with SIF for 0.5, 1, 5, 10, 15, 30 and 60 min.

cellular proliferation (≥ 3 times of control) in 8 patients sensitized to blackgram (Fig. 4b). OD values ranged from 0.088 to 0.577 (mean OD 0.421 ± 0.09) on stimulation with purified protein as compared to 0.323–0.699 with blackgram extract. However, no stimulation (mean OD 0.06 ± 0.01) was observed in non allergic individuals' PBMCs.

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

Biochemical characterization

Digestion in SGF and SIF

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

Periodate treatment

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

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

N-terminal sequencing

N-terminal sequence of the 28 kDa purified protein showed 12 residues GRREDDYDNLQL. In BLAST database, a stretch of residues 'DDYDNLQL' showed homology with Rho-specific inhibitor of transcription termination ($E = 0.42$, Identity = 87%) and NBS-LRR type disease resistant protein from peanut (*Arachis hypogaea*) ($E = 2$, Identity = 77%).

Discussion

The knowledge of biochemical and immunologic properties of disease-eliciting proteins is essential for the appropriate diagnosis and therapy of type I allergic disorders. Diagnosis of allergy with

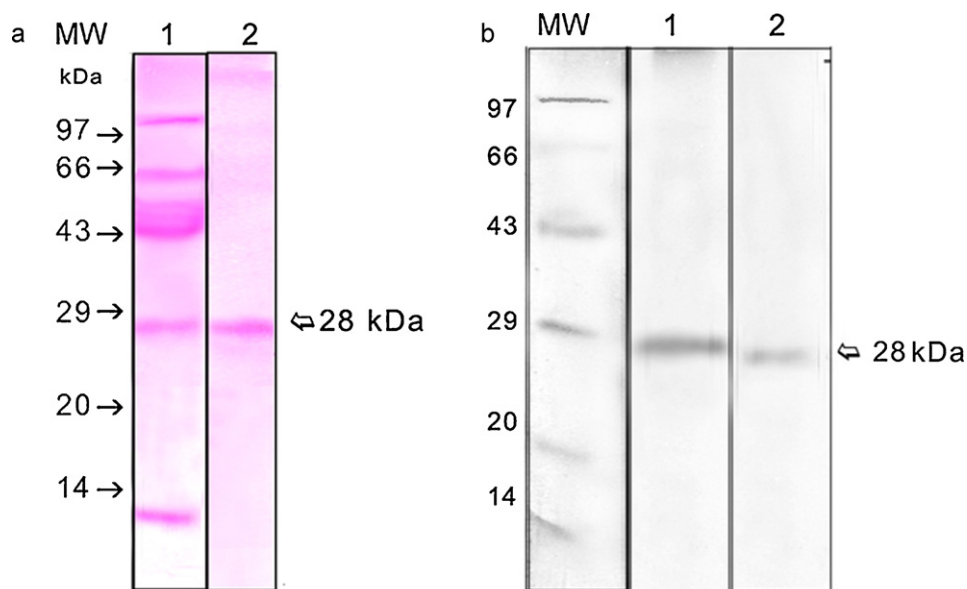


Fig. 6. (a) Glycoprotein detection by Periodic Acid Schiff's (PAS) staining. Lane 1, blackgram extract; lane 2, purified protein. (b) Immunoblot analysis of purified protein after periodate treatment. The 28 kDa protein was electro-transferred onto NCM strip. It was incubated in dark with 20 mM sodium metaperiodate, overnight at 4 °C. The periodate was inactivated for 5–10 min by adding ethylene glycol. To this, 1 mg/ml of sodium borohydride was added and kept overnight at 40 °C. The reaction was stopped by adding a drop of acetic acid. The strips were washed, blocked with 3% defatted milk and immunoblotted with pooled patients' sera. Lane 1, 28 kDa protein (untreated); lane 2, 28 kDa protein after periodate treatment.

purified allergens offers high sensitivity and specificity (Gamboa et al. 2009). Also purified well characterized allergens can facilitate in deciphering the mechanism underlying immunotherapy and to develop patient-tailored therapeutic and prophylactic strategies (Valenta and Niederberger 2007). Legumes are widely consumed as a rich source of dietary proteins but they are implicated in IgE mediated food allergy in predisposed individuals (Ibanez Sandin et al. 1999; Bock et al. 2001; Rougé et al. 2010). Blackgram, a commonly consumed legume has been recognized as one of the important sensitizers among allergic patients in India. IgE binding components of blackgram were identified by immunoblotting with hypersensitive patients' sera (Kumari et al. 2006). In the present study, a 28 kDa allergen was isolated from blackgram extract and characterized by standard *in vivo* and *in vitro* methods.

Over the years, allergenic proteins have been isolated and characterized from food sources using different chromatography and immuno-biochemical techniques. In peanut, 8 allergenic proteins have been identified namely, Ara h 1–Ara h 8. Ara h 1 (63 kDa) was purified on a Mono Q column (Burks et al. 1991) whereas Ara h 2 (19 kDa) and Ara h 3 were purified by High Q anion column (Burks et al. 1992; Koppelman et al. 2003). Gly m Bd 28K – a major allergen from soy, was purified by immunoaffinity column using mAb as a ligand (Tsuji et al. 1997). Anion-exchange chromatography on a Mono Q column and reverse-phase HPLC on a Vydac-C4 column were used to purify Len c 1.01 – a major allergen from lentil. Another 12–16 kDa allergen (Len c 1.02) was purified from boiled lentil by a combination of gel filtration, cation exchange chromatography and reverse-phase HPLC (Sanchez-Monge et al. 2000). In the present study, we have purified a protein of blackgram using a combination of Q-sepharose column and phenyl sepharose column. The protein appeared as a single band of 28 kDa on 12% SDS-PAGE.

An allergen is defined by its ability to exhibit IgE binding and to cross-link IgE/FcεRI on the surface of mast cells and basophils and subsequent mediator release (Kay 2001, 2002). In the present study, allergenicity of the purified 28 kDa protein was assessed by skin prick test, specific IgE, ELISA inhibition, immunoblotting and histamine release assay. Of the 9 patients SPT positive to blackgram,

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

Resistance to proteolytic enzymes and heat is a property associated with many food allergens. These produce stable allergen fragments that resist conditions of the gastrointestinal tract, and have the potential to sensitize the immune system. Astwood et al. (1996) showed that food allergens were either resistant to pepsin in SGF or formed stable fragments, whereas the nonallergens were completely digested within 15 s. Stability of the whole protein or fragments from the allergens tested ranged from 8 to 60 min in SGF. Digestion of Ara h 1 by pepsin showed IgE reactive peptide fragments of 16–29 kDa that were stable till 30 min to 3 h whereas treatment with trypsin and chymotrypsin resulted in IgE reactive fragments ranging from 16 to 59 kDa (Maleki et al. 2000). In the present study, treatment of 28 kDa protein, with pepsin resulted in stable IgE reactive fragments. The 28 kDa protein however digested within 15 min upon treatment with pancreatin. Pancreatin has been a mixture of amylase, trypsin and lipase with trypsin being the only proteolytic component which is assumed to digest rest of the constituent proteins (amylase and lipase). This explains the

reduction in number of bands in the molecular weight range of 29–27 kDa with time.

The 28 kDa protein was detected as a glycoprotein by PAS staining. On periodate treatment, there was a decrease in IgE binding with blackgram hypersensitive patients' pooled sera. Ara h 1, a major allergen of peanut is a glycoprotein belonging to the vicilin family (Shin et al. 1998). Len c 1 from lentil also showed 3 isoforms varying in their N-glycosylation (Lopez-Torrejon et al. 2003). The major food allergens identified as class 1 allergens are water-soluble 10–70 kDa glycoproteins such as caseins (milk), ovomucoid (egg), nonspecific lipid transfer proteins like Mal d 3 (apple) and Zea m 14 (corn) (Breiteneder and Mills 2005).

IL-4 cytokine is essential for initiation and maintenance of the allergic disease (Seder et al. 1991; Scott-Taylor et al. 2005). Allergen-dependent production of T_H2 cytokines (IL-4, IL-5, IL-13) have already been reported in many previous studies (Kurup et al. 2002; Kikuchi et al. 2006; Schwab et al. 2003) which are responsible for inflammation and other immunopathological events. Food allergenicity is characterized by abnormal IgE production, peripheral eosinophilia, mast cell activation, and induction of T_H2 lymphocytes expressing cytokines such as IL-4 and IL-10 (Wong et al. 2001; Wierenga et al. 1991). In the present study, the purified protein induced significant lymphocyte proliferation and increased secretion of IL-4 than controls. The data indicates the predominant T_H2 response on exposure to purified protein further suggesting its allergenicity. IFN- γ levels were however observed low (20–50 pg/ml) as compared to non-allergic controls on stimulation with both blackgram extract and or purified protein (data not shown).

The N-terminus-12 residues sequence of 28 kDa purified protein obtained was GRREDDYDNLQL. N-terminal sequence showed homology with NBS-LRR type disease resistant protein from peanut (*Arachis hypogaea*) and Rho-specific inhibitor of transcription termination.

In conclusion, a 28 kDa protein was purified and characterized as a major allergen from blackgram. The purified protein seems a potent allergen and may be implicated in possible strategies for component resolved diagnosis and therapy.

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