



Purification, characterization and allergenicity assessment of 26 kDa protein, a major allergen from *Cicer arietinum*



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ABSTRACT

Chickpea (CP), a legume of the family Fabaceae, is an important nutrient-rich food providing protein, essential amino acids, vitamins, dietary fibre, and minerals. Unfortunately, several IgE-binding proteins in CP have been detected that are responsible for allergic manifestations in sensitized population. Therefore, the prevalence of CP induced allergy prompted us towards purification, characterization and allergenicity assessment of a major ~26 kDa protein from chickpea crude protein extract (CP-CPE). Purification of CP 26 kDa protein was done using a combination of fractionation and anion exchange chromatography. This protein was further characterized as “Chain A, crystal structure of a plant albumin” from *Cicer arietinum* with Mol wt 25.8 kDa by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Further, allergenic potential of purified 25.8 kDa protein was assessed using *in vivo* and *in vitro* model. Purified protein showed IgE-binding capacity with sensitized BALB/c mice and CP allergic patient's sera. Enhanced levels of specific and total IgE, MCP-1, MCPT-1, myeloperoxidase, histamine, prostaglandin D2, and cysteinyl leukotriene were found in sera of mice treated with CP ~26 kDa protein. Further, expressions of Th2 cytokines (i.e. IL-4, IL-5, IL-13), transcription factors (i.e. GATA-3, STAT-6, SOCS-3) and mast cell signaling proteins (Lyn, cFgr, Syk, PLC-γ2, PI-3 K, PKC) were also found increased at mRNA and protein levels in the intestines of mice treated with CP ~26 kDa protein. In addition, enhanced release of β-hexosaminidase, histamine, cysteinyl leukotriene and prostaglandin D2 were observed in RBL2H3 cell line when treated (125 μg) with CP 26 kDa protein. Conclusively, *in vivo* and *in vitro* studies revealed the allergenic potential of purified CP 26 kDa protein. Being a potential allergen, plant albumin may play a pivotal role in CP induced allergenicity. Current study will be helpful for better development of therapeutic approaches to prevent the allergenicity in CP sensitized individuals.

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1. Introduction

Allergic reactions are consequences of abnormal immune response to common and harmless substances, present in the environment. A substance that causes allergic reaction is known as allergen. In last two decades, the prevalence and severity of allergic diseases are increasing worldwide and food allergy con-

stitutes a major part of this increase (Sicherer and Sampson, 2010). Generally, the food allergy may be defined as an adverse health effect that arises from a specific immune response on exposure to a given food (Boyce et al., 2010). Moreover, food induced allergy is characterized by induction of Th2 cells and production of IL-4 cytokine that further acts upon naive T-cells and causes their differentiation into allergen specific Th2 cells to secrete Th2 cytokines including IL-4, IL-5, IL-13 which assist B-cell responses and induce the production of allergen specific IgE (McKenzie et al., 1998). Further, various transcription factors like GATA-3, STAT-6, SOCS-3, C-MAF also play an important role in skewing of immune response towards Th2 reactions thereby provoking allergic manifestations by enhancing the production of Th2 cytokines (Kumar et al., 2012).

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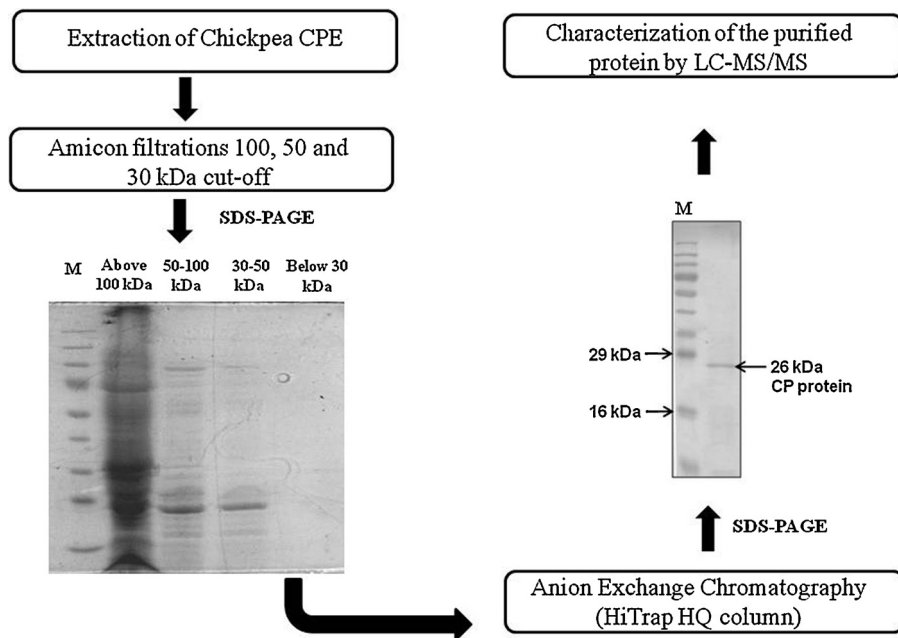


Fig. 1. Diagrammatic representation of purification and characterization of 26 kDa protein from CP-CPE.

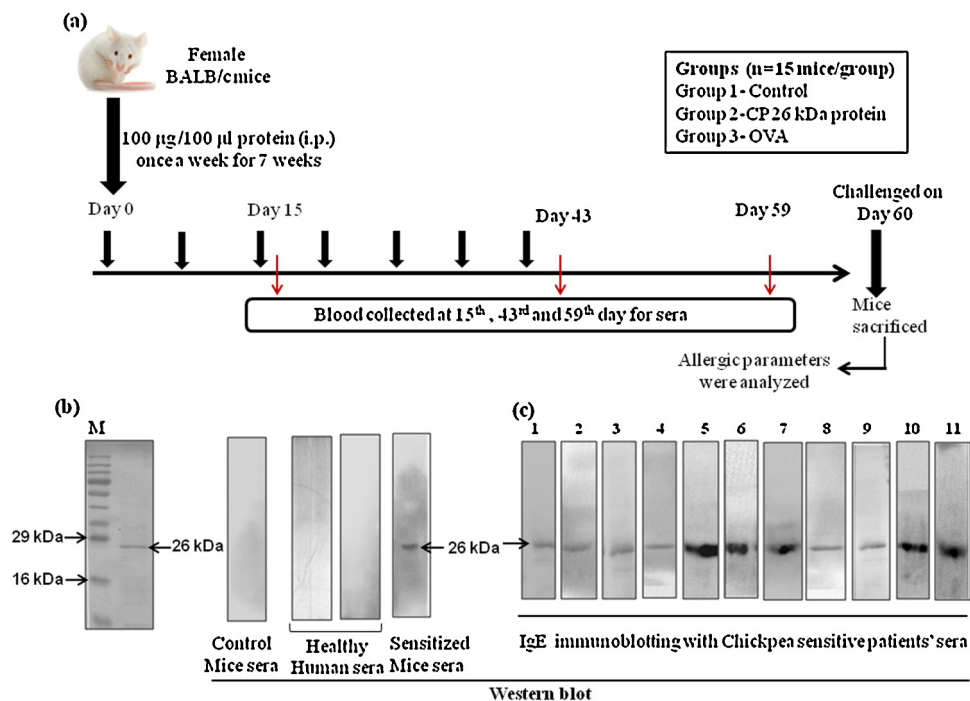


Fig. 2. (a) Diagrammatic representation of animal treatment protocol (b) SDS-PAGE of CP 26 kDa protein and IgE immunoblot with PBS treated mice and healthy humans' sera. No IgE-binding were found with control mice and healthy human sera (c) IgE immunoblot showing IgE-binding capacity of CP 26 kDa protein with sensitized mice and CP allergic patients' sera.

The most common allergenic foods include milk and dairy products, egg, gluten containing cereals (e.g., wheat, rye, barley), fish, crustaceans, shellfish and legumes (e.g., peanut, soybean) (Špičák, 2010). Legumes are essential constituent of human diets as they are rich source of proteins and oils but unfortunately, legumes are also important source of allergens. The legume allergens are mainly associated with four protein families (i) Cupins (ii) Prolamins (both included in seed storage proteins), (iii) Profilins and (iv) Pathogen

related proteins. In recent years, the major allergens from different legumes have been identified and characterized including peanut (Ara h1–Ara h7), soybean (Gly mBD), green gram (Vig r2–Vig5), red gram (Caj c1–Caj c6) and green bean (Pha v3) that are responsible for IgE-mediated allergic reactions in susceptible individuals (Verma et al., 2013). Among leguminous crops, chickpea (CP) is also one of the most commonly consumed legume, particularly in the populations of developing nations, and also in mediterranean

areas as it is a good source of protein, soluble and insoluble fibres. Mature chickpea grains contain 60–65% carbohydrate, 6% fat, and 17% protein (Gopalan et al., 1989). However, allergenic potential of CP has not been explored well. Therefore, it is important to purify and explore the allergenic potential of IgE-binding CP proteins to understand their distinct role in allergy.

In the earlier study, it was reported that CP is able to cause IgE-mediated allergic reactions in nasobronchial allergic patients and sensitized BALB/c mice, as CP-CPE contains seven IgE-binding proteins in the range of 15–95 kDa (Verma et al., 2012). However, purification and characterization of major CP allergens are necessary to explore the extent of allergenicity caused by purified allergens. Therefore, the aim of the present study was to purify the IgE-binding protein from CP-CPE by using column chromatography and further characterization by Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. In the current study, purification of CP 26 kDa protein was done as this protein showed the IgE-binding property with all screened CP sensitive patients. So, we decided to purify this 26 kDa major allergenic protein from CP and assessed the allergenic potential of this protein *in vitro* as well as *in vivo* situations.

2. Materials & methods

2.1. Ethical permissions

Human studies were carried-out with the informed patient's consent and the study protocol was approved by the human ethics committee of the King George Medical University (KGMU), Lucknow, India (Ref: IEC No. 2938/R. Cell-11). Animal studies were carried out after approval by an Institutional Animal Ethics Committee of CSIR- Indian Institute of Toxicology Research (IITR), Lucknow, India (IITR/IAEC/27/11-05/2011).

2.2. Skin prick test and blood collection

Skin prick test (SPT) was performed according to earlier described method (Verma et al., 2012) with allergen kit (Alcit India Private Limited) on allergic patients (n=200) and normal healthy individuals (n=10) at the Department of Respiratory Medicine, King George Medical University (KGMU), Lucknow, India. The patients were mainly suffering from Bronchial Asthma, Allergic Rhinitis, Dermatitis and Urticaria (Verma et al., 2012). Weal diameter equal to positive control or more were considered as marked positive skin reactions. If smaller weal as compared to positive control then +1, equal +2, larger 3 and for bigger with pseudopodia then +4.

2.3. Chickpea crude protein extraction

Chickpea seeds (Pusa-1003 variety) were purchased from a local certified seed vendor and the same lot was used throughout the study. CP-CPE was prepared by previously described method (Misra et al., 2009) (Supplementary Data S1). The Limulus amoebocyte lysate (LAL) assay was performed to detect endotoxin contamination in CP-CPE and purified 26 kDa protein with the Pierce LAL Chromogenic Endotoxin Quantification kit (PI- 88282, Thermo Fisher Scientific, USA) according to the manufacturers' protocol.

2.4. Purification and characterization of the IgE-binding protein of chickpea

The purification of CP protein was carried out by previously described method (Kumar et al., 2014) (Fig. 1). CP-CPE supplemented with 5 mM PMSF (phenylmethylsulfonyl fluoride) (Sigma,

St. Louis, United States), was used to concentrate in Amicon centrifugal tubes (Millipore, USA) in a step wise manner (Amicon Ultra-15, Mr. 100,000 cut-off followed by Amicon Ultra-15, Mr. 50,000 cut-off and further, Amicon Ultra-15, Mr. 30,000 cut-off). Further, the desired fractions were subjected to HiTrap anion exchanger 5.0 ml column (GE Healthcare, Little Chalfont, United Kingdom) using Econo gradient pump and ultraviolet (UV) monitor (Bio-Rad, Hercules, California, U.S.) with a speed of 5 ml/min and gradient solution of Tris and 50 mM NaCl solution (pH 8.02). Fractions obtained, along with a protein ladder range between 250 kDa to 10 kDa (Fermentas, Vilnius, Lithuania) were resolved on a 12% separating gel and stained with G-250 Coomassie Brilliant Blue dye. The fractions were further tested for IgE-binding potential on immunoblot using pooled sera of CP allergic patients. We further intended to characterize the IgE-binding protein of CP using LC-MS/MS as per the earlier described methods (Kumar et al., 2014). The LC-MS/MS studies were carried out at The Centre for Genomic Applications (TCGA), New Delhi, India. Further, bioinformatics analysis was performed using Algpred and SDAP software to predict the possibility of allergenicity of purified 26 kDa protein (Supplementary Data S2).

2.5. Treatment of BALB/c mice with purified CP 26 kDa protein

Healthy 6–8week old female BALB/c mice (18 ± 3 g) were obtained from an animal breeding colony of CSIR-IITR, Lucknow, India. Mice were maintained under standard laboratory conditions in a pathogen free environment and on CP and ovalbumin (OVA) free diet.

Mice (n=15) were sensitized with CP 26 kDa protein and OVA according to the earlier described method (Misra et al., 2011). OVA was taken as positive control. Briefly, BALB/c mice were randomly divided into three groups of 15 mice each. After one week of acclimatization period, group 1, 2 and 3 were given PBS (pH 7.4), CP 26 kDa protein and OVA by intraperitoneal route, respectively. Mice were given 100 µg proteins (CP 26 kDa protein and OVA) in 100 µl PBS once a week for 7 weeks. Blood samples were collected from the retro-orbital sinus to measure immunoglobulins (total/specific IgE, IgG1 antibodies), MCP-1, histamine levels and other allergic parameters in the pooled sera. Sensitized mice were challenged on day 60 with 5 mg/ml CP 26 kDa protein and OVA in respective group. Challenged mice in each group were sacrificed by cervical dislocation. The lungs, intestine and spleen were taken out for histopathological analysis and fixed in 10% formalin in PBS. Animal treatment protocol is illustrated in Fig. 2 (a).

2.6. Immunoblot analysis

To detect IgE-binding capacity of CP 26 kDa protein, IgE immunoblotting was performed according to the earlier described procedure (Misra et al., 2011) (Supplementary Data S3).

2.7. Measurement of rectal temperature, specific/total IgE, monocyte chemotactic protein-1 (MCP-1), mouse mast cell protease 1 (mMCPT-1), and thymic stromal lymphopoietin (TSLP)

Rectal temperature was monitored before and 20 min after challenging the sensitized mice with protein using digital rectal thermometer (Bioseb, France). Specific IgE level against CP 26 kDa protein was estimated using enzyme-linked immunosorbent assay (ELISA) according to the method described earlier (Misra et al., 2010). Levels of total IgE, MCP-1, mMCPT-1, and TSLP in the sera of CP 26 kDa protein treated mice were estimated using ELISA kits (for total IgE and MCP-1: BD Biosciences, San Diego, CA, USA; for mMCPT-1 and TSLP: eBioscience, San Diego, CA, USA) as per man-

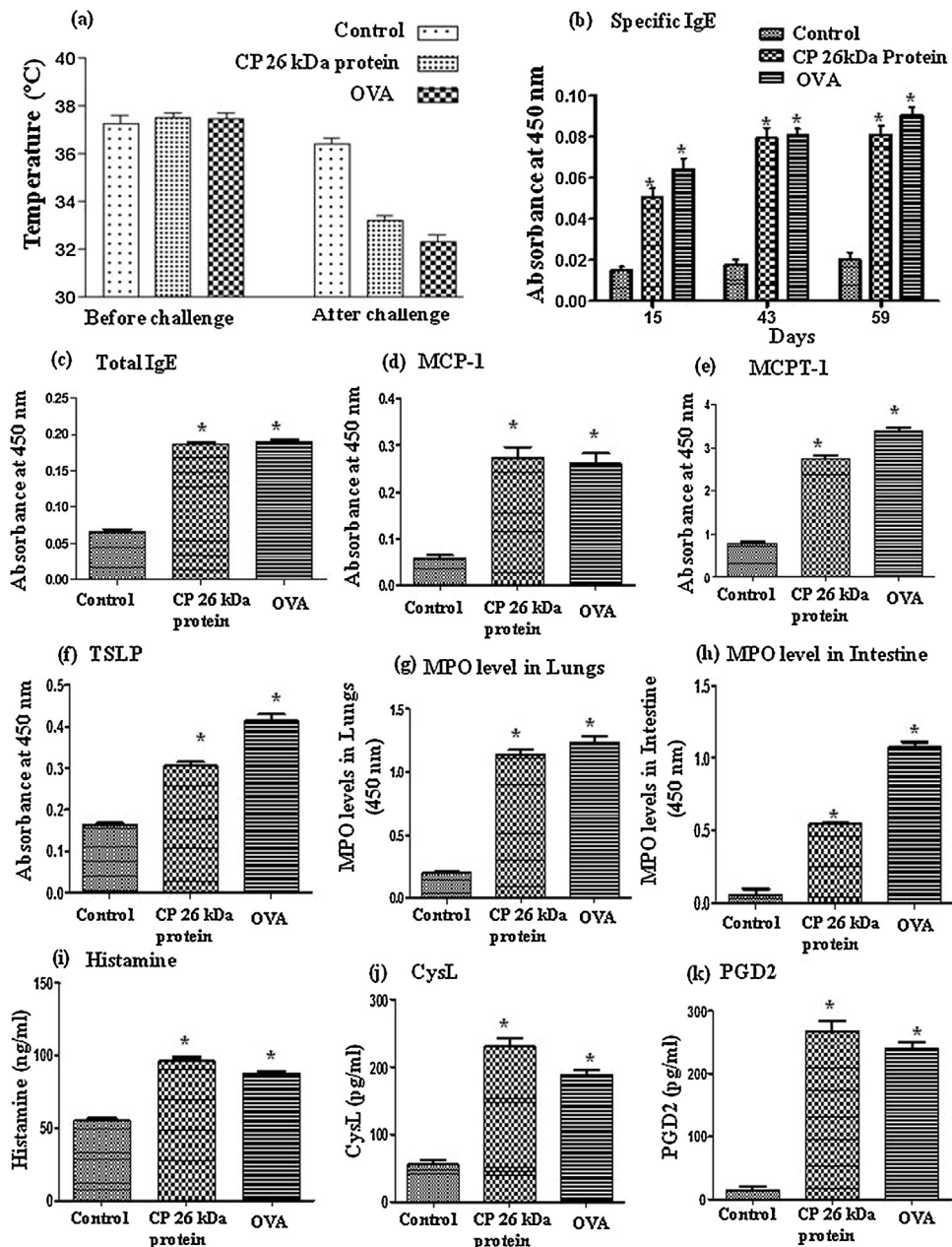


Fig. 3. (a) Rectal temperature was measured on day 60 before and 20 min after challenge. Estimation of (b) specific IgE, (c) total IgE, (d) MCP-1, (e) MCPT-1, and (f) TSLP levels in sera of mice sensitized with CP 26 kDa protein and OVA by ELISA. Enhanced levels of these allergic mediators were evident in sera of mice treated with CP 26 kDa protein, (g–h) Evaluation of Myeloperoxidase (MPO) levels in lungs, and intestine of control, CP 26 kDa protein and OVA treated mice. Serum levels of (i) Histamine, (j) CysL and (k) PGD2 in CP 26 kDa protein and OVA treated mice. Elevated levels of these markers of allergic reaction were seen in CP 26 kDa protein treated mice as compared to control group indicating its allergy provoking potential. All data are presented in mean $\pm$ SEM; (* p < 0.05) when compared with control.

ufacturers' instructions. The results were expressed in terms of absorbance.

2.8. Myeloperoxidase (MPO) assay

Intestine and lungs were cleaned thoroughly with cold normal saline. MPO level was determined according to a previously described method (Cardoso et al., 2009) (Supplementary Data S4).

2.9. Measurement of histamine, cysteinyl leukotriene (CysL), prostaglandin D2 (PGD2) in sensitized mice sera

Histamine, CysL and PGD2 levels in sera of CP 26 kDa protein treated mice were determined using EIA kit (for histamine: SPI-

BIO, Montigny le Bretonneux, France; for PGD2 and CysL: Cayman chemicals, USA) following manufacturers' protocol.

2.10. Mediators release from RBL-2H3 cells

RBL-2H3 cells (Rat basophilic leukemia cells) were obtained from the American Type Cell Culture (ATCC, Manassas, VA, USA). The passive sensitization of RBL-2H3 cells with CP 26 kDa protein treated mice sera was carried out according to the method described earlier (Diesner et al., 2008). The release of β -hexosaminidase following exposure of 25, 50, 75, 100 and 125 μ g CP 26 kDa protein was calculated as the method described earlier (Kumar et al., 2014). The levels of histamine, CysL and PGD2 were estimated by commercially available ELISA kits (for histamine: SPI-

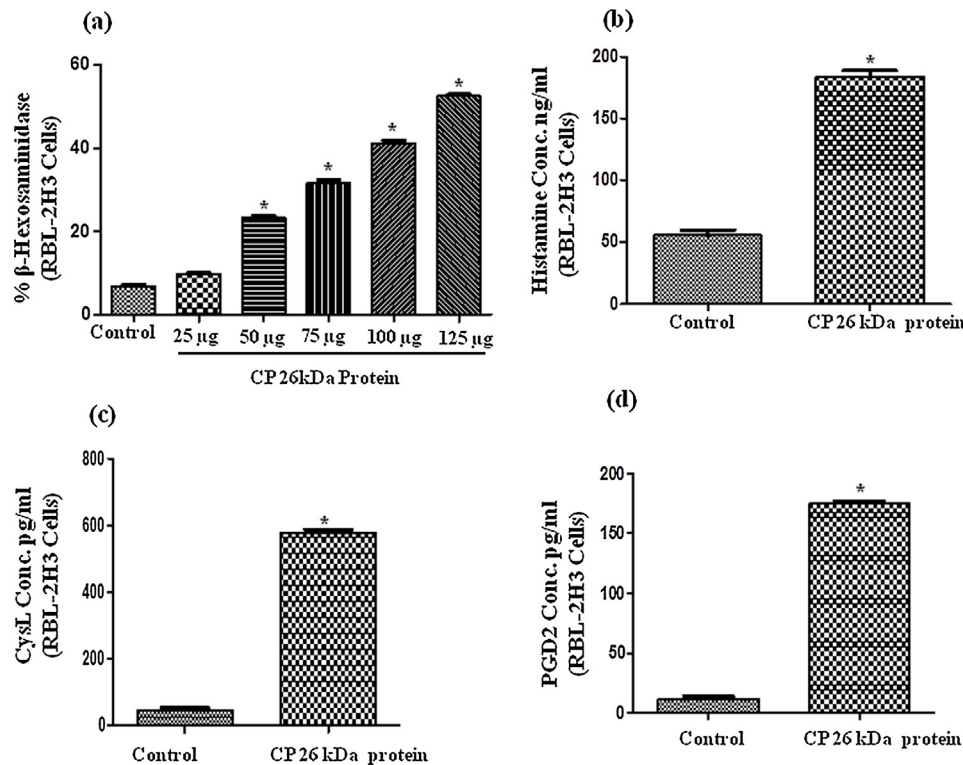


Fig. 4. (a) CP 26 kDa protein induced β -hexosaminidase release in a dose dependent manner in RBL-2H3 cells. Maximum release was observed at 125 μ g dose, (b) Histamine release, (c) CysL release and (d) PGD2 release at 125 μ g dose from RBL-2H3 cells after sensitization with pooled sera of CP 26 kDa protein treated mice. Data are presented in mean $\pm$ SEM (* $p < 0.05$) when compared with control.

BIO, Montigny le Bretonneux, France; for PGD2 and CysL: Cayman chemicals, Ann Arbor, Michigan, USA).

2.11. Histopathological studies

The intestine, lungs and spleen were taken for histological analysis from control, CP 26 kDa protein and OVA treated mice. Tissue sections were stained with hematoxylin and eosin for microscopic examination (125 $\times$ and 500 $\times$ magnifications).

2.12. Immunohistochemistry for eosinophil levels, GATA-3 and T-bet expressions

GATA-3, T-bet expressions and eosinophil count in the intestine, lungs and spleen sections were detected by immunohistochemistry according to the earlier described method (Li et al., 1995). Briefly, sections of tissues (3–5 μ m) from different groups were incubated with rabbit anti mouse eosinophils major basic protein (EMBP), rabbit anti mouse GATA-3 and T-bet antibodies respectively (Santa Cruz biotechnology, CA, USA) that were used as primary antibodies, followed by HRP conjugated anti-rabbit IgG used as secondary antibody. 3, 3'-Diaminobenzidine [(DAB), Sigma Chemical Company, St. Louis, MO, USA] staining followed by counter stain with hematoxylin was done and the images were captured using a microscope (Nikon Eclipse TE2000-S).

2.13. Semi quantitative polymerase chain reaction (PCR)

A semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of Th2 cytokines (IL-4, IL-5, IL-13) and Th2/Th1 transcription factors (GATA-3, and T-bet) in the intestine of PBS, CP 26 kDa protein and OVA treated groups were carried out by gene specific primers according to the earlier described method (Kumar et al., 2014) (Supplementary Data S5).

Primers were designed by using Primer 3 software (<http://seqtool.sdsc.edu/CGI/BW.cgi>). The primer sequences were as follows:

IL-4: sense 5'-TCGGCATTGTAACGAGGTC-3', anti-sense 5'-AAAAGCCCCGAAAGAGTCT C-3'; IL5: sense 5'-TCACCGAGCTCTGTTGACAA-3', anti-sense 5'-CCACACTTCTCTTTTGGCG-3'; IL-13:sense5'-GACCCAGAGGATATTCATG-3',anti-sense 5'-CCAGCAAAGTCTGATGTGAG-3'; GATA-3:sense 5'-TCTCACTCTCGAGGCAGCATGA-3', anti-sense 5'-GGTACCATCTCGCCGCCACAG-3'; T-bet: sense 5'-TCCCATTCTCTGCTCTCA-3', anti-sense 5'-GCTGCCTTCTGCCTTTC-3'; GAPDH: sense 5'-TTCACCACCATGGAGAAGGC-3, anti-sense 5'-GGCATGGACTGTGGTCATGA-3'.

2.14. Western blots for transcription factors

The levels of Th1/Th2 transcription factors GATA-3, SOCS3, C-MAF, STAT6, NFAT, T-bet in the intestinal proteins of control, CP 26 kDa protein and OVA treated mice were detected by immunoblotting according to previously described method (Kumar et al., 2014) (Supplementary Data S6)

2.15. Western blots of mast cells signaling molecules

We further studied the role of CP 26 kDa protein induced mast cell activation and signaling. For this study, we have carried out western blot analysis of signaling molecules Fc ϵ R1, p-Lyn, p-cFgr, p-Syk, p-PLC- γ 2, PI3 K and p-PKC using specific antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) of mast cells. The β -actin was used as internal control.

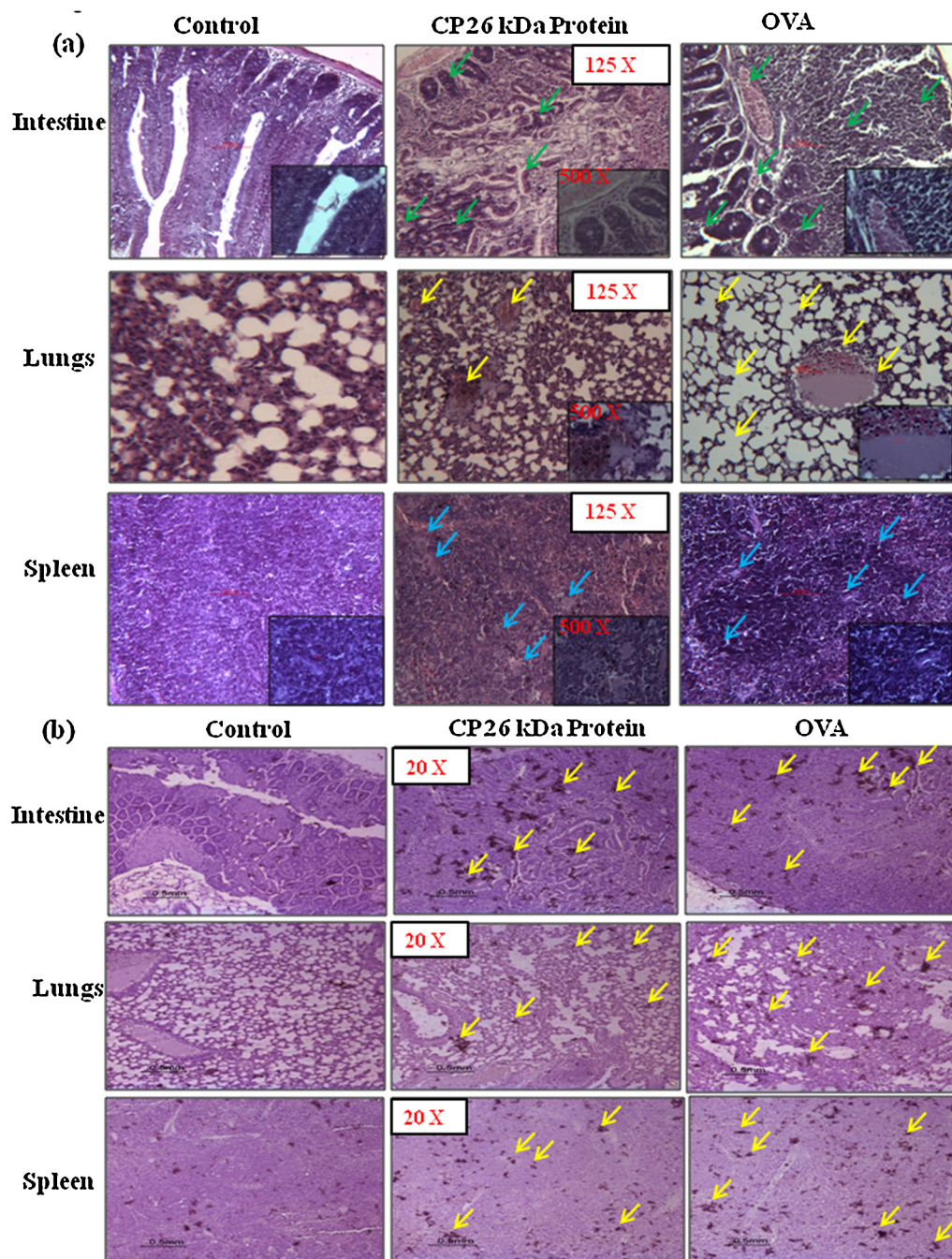


Fig. 5. (a) Histopathological analysis of intestine, lungs and spleen of control, CP 26 kDa protein and OVA treated mice groups. Intestines of treated groups showed exfoliation of villi with infiltration of inflammatory cells (indicated with green arrow), lungs showed hyperplasia of bronchial lining epithelium and thickening of alveolar septa (indicated with yellow arrow), spleen depicted megakaryocytes and lymphoid hyperplasia (indicated with blue arrow). Histological images has been taken at 125 $\times$ and 500 $\times$ for better clarity (b) Immunohistochemical analysis for eosinophils in intestine, lungs and spleen of control, CP 26 kDa protein and OVA treated groups. Significant increase in eosinophil count was observed in intestine, lungs and spleen of CP 26 kDa protein and OVA treated groups when compared to control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.16. Statistical analysis

The data were expressed as mean $\pm$ SEM. All the data were analyzed using Instat version 5.0 (GraphPad, San Diego, CA, USA; <http://www.graphpad.com>). Differences between values were compared by using one-way ANOVA with Bonferroni as a post hoc test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. CP purified protein was characterized as albumin

CP purified protein was subjected to LC–MS/MS analysis and characterized as Chain A, crystal structure of a plant albumin from *Cicer arietinum* with Mol wt 25.8 kDa (pI-8.24, score 201). Algpred software result indicated that purified protein can be an allergenic protein on the basis of hybrid approach. Moreover, allergenicity

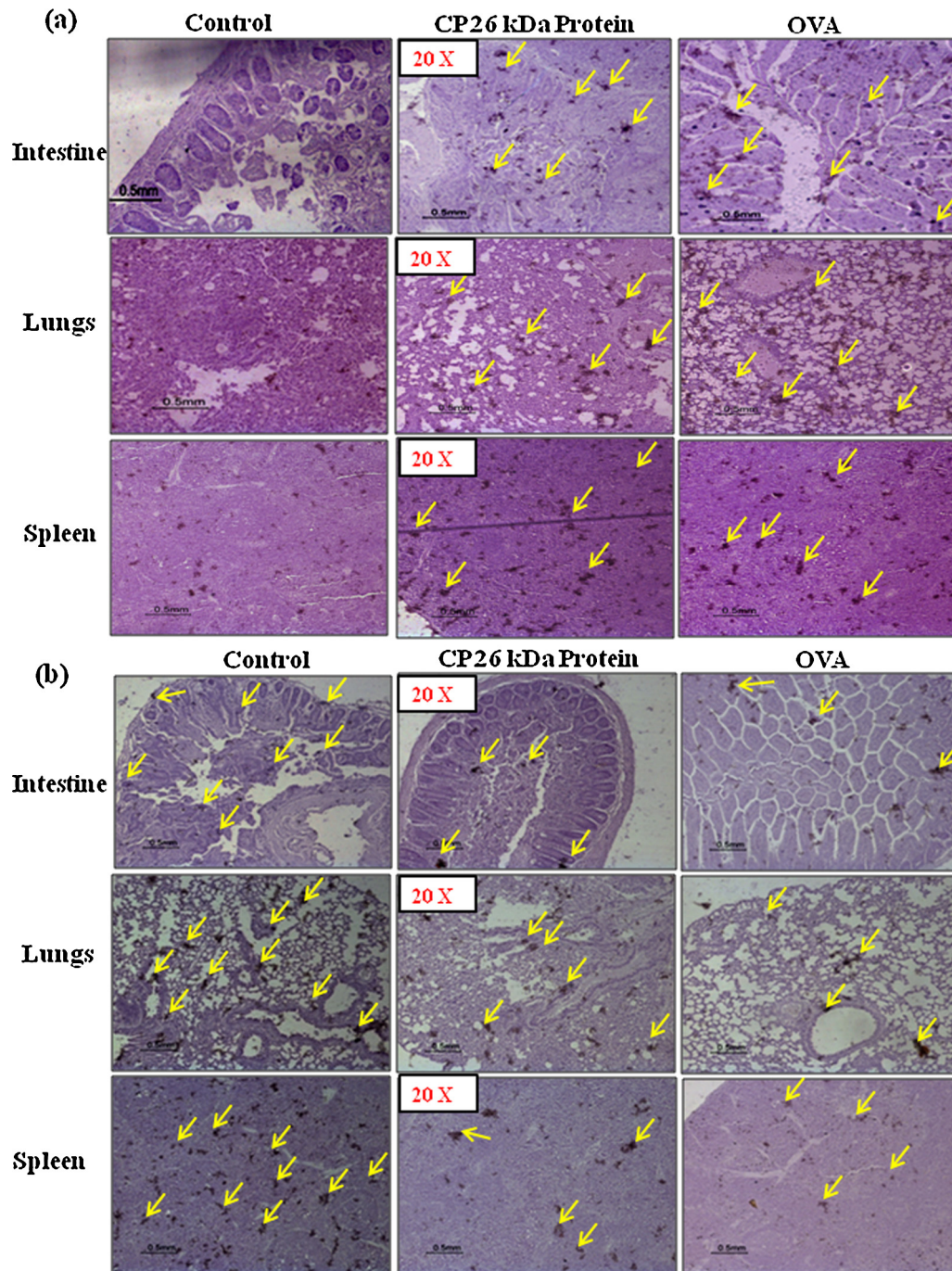


Fig. 6. Immunohistochemistry for GATA-3 expression (Th2 transcription factor) in intestine, lungs and spleen of control, CP 26 kDa protein and OVA treated groups. Enhanced expression of GATA-3 was observed in intestine, lungs and spleen of CP 26 kDa protein and OVA treated groups when compared to control that indicate towards Th2 response in mice treated with CP 26 kDa protein (b) Immunohistochemistry for T-bet expression (Th1 transcription factor) in intestine, lungs and spleen of control, CP 26 kDa protein and OVA treated groups. Down regulated expression of T-bet was observed in intestine, lungs and spleen of CP 26 kDa protein and OVA treated groups when compared to control.

prediction by SDAP database showed matching with known allergens of green gram Vig r 4 with 55.07% similarity (Supplementary Table 1 & 2).

3.2. Purified CP 26 kDa protein showed IgE-binding property

The IgE-binding property of CP purified protein was confirmed by IgE Immunoblot using pooled sera of sensitized mice and CP allergic patients. No IgE-binding was detected with the healthy individual's sera as well as control mice sera (Fig. 2b.). We fur-

ther extended our experiment to assess the allergenic potential of purified protein using *in vivo* and *in vitro* approaches.

3.3. Body temperature in sensitized mice after challenge and measurement of other allergic parameters in mice sera

Drop in body temperature in the range of 3–5 °C was found in mice 20 min after challenge with CP 26 kDa protein. No such anaphylaxis symptoms were observed in the control group (Fig. 3 a). The group sensitized with CP 26 kDa protein showed significantly

higher levels of specific IgE and total IgE as compared to control group (Fig. 3b and c). Moreover, increased levels of MCP-1, MCPT-1 and TSLP were evident in CP 26 kDa protein treated mice sera as compared with the control group (Fig. 3d–f). Further, significantly increased levels of MPO in the lungs and intestine of CP 26 kDa protein treated mice group was observed as compared to control group (Fig. 3g and h). Analysis of histamine, CysL and PGD2 indicates higher levels in the sera of CP 26 kDa protein treated mice when compared to control group (Fig. 3i–k). OVA treated mice group used as positive control in the study, had shown increased levels of all allergic parameters as compared to control group and induced allergenic response was more or less similar to CP 26 kDa protein.

3.4. Mediators release from RBL-2H3 cells following CP 26 kDa protein exposure

We next sought to explore the allergic potential of CP 26 kDa protein using RBL-2H3 cells as an *in vitro* tool. CP 26 kDa protein exposed RBL-2H3 cells showed 9, 23, 31, 41 and 52% increase in the release of β -hexosaminidase at the concentrations of 25, 50, 75, 100 and 125 μ g when compared to control (Fig. 4a). Furthermore, dose dependent release of β -hexosaminidase from RBL-2H3 cells was found maximum at the dose of 125 μ g. Therefore, at this particular concentration, we further analyzed the levels of histamine, CysL and PGD2 in culture supernatant of RBL-2H3 cells. Enhanced release of histamine, CysL and PGD2 were evident in supernatants of CP 26 kDa protein treated RBL-2H3 cells as compared to control (Fig. 4b–d).

3.5. Histopathological analysis of the intestine, lungs and spleen

Prominent histopathological changes in intestine, lungs and spleen in CP 26 kDa protein treated mice were found. Destruction of mucosal lining and inflammatory cell infiltration were observed in the intestine of CP 26 kDa protein treated mice. The lungs showed hyperplasia and exfoliation of bronchial lining epithelium and thickening of alveolar septa. The spleen depicted lymphoid hyperplasia. Large sized megakaryocytes were also observed in treated group. Control group mice showed normal structure of the lungs, intestine and spleen (Fig. 5a). Histopathological analysis of OVA treated mice showed changes similar to CP 26 kDa protein treated group with more severe allergic symptoms in intestine, lungs and spleen.

Further, analysis of eosinophil count indicated the increased number of eosinophils in the intestine, lungs and spleen of CP 26 kDa protein treated group as compared to the control group. The elevated level of eosinophils in CP 26 kDa protein sensitized mice was found similar to OVA treated mice group (Fig. 5b).

3.6. GATA-3 and T-bet expression in lungs, spleen and intestine

The GATA-3 levels were significantly up regulated in intestine, lungs and spleen following CP 26 kDa protein exposure as compared to control (Fig. 6a). The levels of T-bet were found to be decreased in all the organs taken from CP 26 kDa protein treated mice group as compared to control (Fig. 6b).

3.7. Expression of Th2 cytokines and transcription factors at m-RNA level

The mRNA levels of Th2 cytokines (IL-4, IL-5 and IL-13) were found up-regulated in the intestine of CP 26 kDa protein treated group when compared to their respective controls.

Enhanced GATA-3 expression and down regulated T-bet expression in CP 26 kDa protein treated group was observed when compared to control group (Fig. 7).

3.8. Western blot analysis of transcription factors

Higher expressions of NFAT, STAT-6, C-MAF, GATA-3, SOCS-3, and comparatively lower expression of T-bet were observed in intestinal proteins of the CP 26 kDa protein treated mice in comparison to control group. Expression level of NFAT was 2.3 fold, STAT-6 was 2.8, C-MAF was 3.2, GATA-3 was 2.6 and SOCS-3 was 0.4 fold higher in CP 26 kDa protein treated group when compared to control. The expression level of T-bet was approximately 0.6 fold lower in CP 26 kDa protein group as compared to control. β -Actin was taken as endogenous control (Fig. 8).

3.9. Western blot analysis for signaling molecules

Western blotting analysis revealed significantly increased levels of kinases and adaptor molecules like p-Lyn, p-cFgr, p-Syk, p-PLC- γ 2, PI-3 K, p-PKC in the CP 26 kDa protein treated group that are involved in mast cell activation and degranulation (Fig. 9). These western blot results confirm that CP 26 kDa protein has the ability to cross link with IgE bound to mast cells and activates a signaling cascade within mast cells that leads to their degranulation and release of allergic mediators.

4. Discussion

Chickpea (CP) is an important legume consumed worldwide due to its nutritious nature. It was previously reported that CP-CPE contains several allergic proteins (Niphadkar et al., 1997; Martinez et al., 2000; Verma et al., 2012). In our earlier study, we have identified seven IgE-binding proteins in the range of 15–95 kDa but the purification and characterization of CP allergens is yet to be explored. The main focus of the present study was to purify CP 26 kDa protein and further assess its allergic potential via applying different scientific approaches.

The bioinformatics approach using SDAP analysis indicated the similarity of CP 26 kDa protein with green gram allergen Vig r 4. When tested through Algpred software, this purified protein qualified as potential allergen in some criteria which suggest that further experimental evidence are required to confirm the allergenicity of CP 26 kDa protein.

In the series of experiments, IgE immunoblotting of CP 26 kDa protein was performed which showed strong IgE-binding with sera of CP sensitized mice as well as CP allergic patients. This result indicate that it is one of the major allergen of CP-CPE. We next sought to assess the allergenic potential of CP 26 kDa protein in female BALB/c mice because they readily display Th2 responses and have the capacity to produce IgE antibody (Helm, 2002). Significantly higher levels of total and specific IgE were evident in sera of CP 26 kDa protein treated mice confirming its potential to cause allergic symptoms in murine model. Further, we observed that CP 26 kDa protein sensitized mice when challenged with high dose of protein showed symptoms of anaphylaxis including drop in body temperature. Our result is in agreement with the study of Li et al. (2000), revealed that mice orally sensitized with peanut protein showed symptoms of anaphylaxis after challenge with high dose of the same protein (Li et al., 2000). Further, we analysed the levels of some important chemokines including MCP-1, MCPT-1 and TSLP that play an important role in the induction of Th2 response. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that plays an important role in monocyte and CD4⁺/CD8⁺ lymphocyte recruitment. In addition, MCP-1 also acts as a potent histamine-releasing agent for basophils and mast cells (Gunn et al., 1997; Conti et al.,

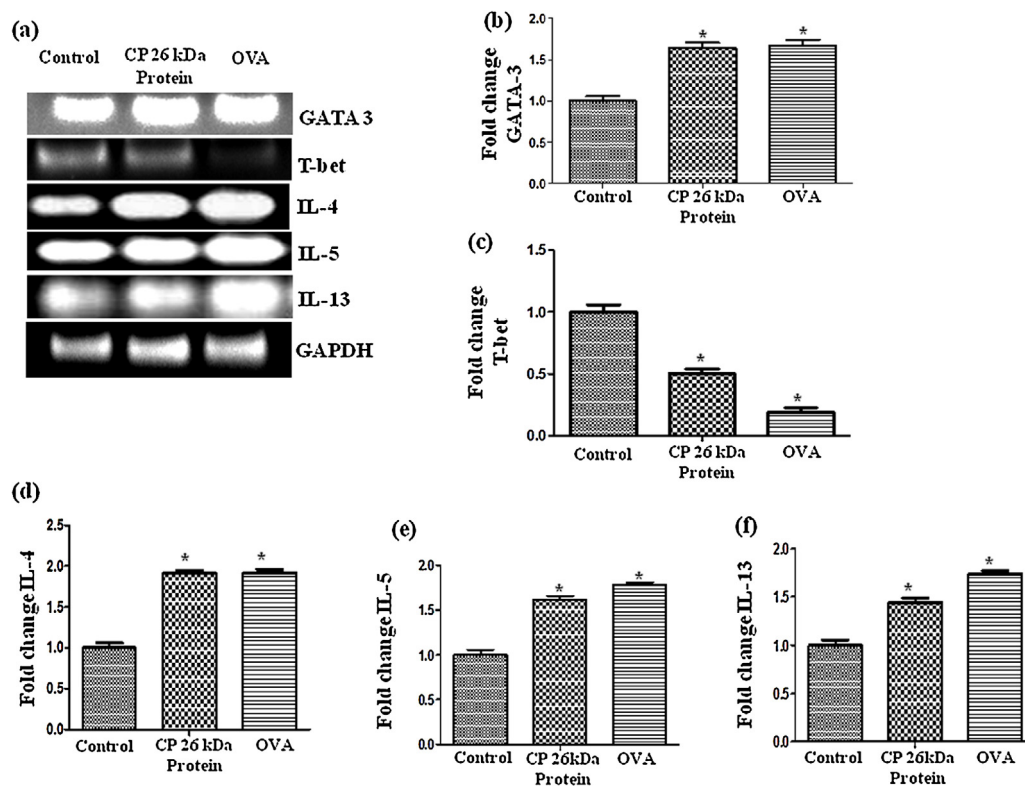


Fig. 7. Semi quantitative polymerase chain reaction (PCR) for gene expressions. (a) The mRNA expressions of cytokines and transcription factors in the intestine of control, CP 26 kDa protein and OVA groups and densitometry of (b) GATA-3, (c) T-bet, (d) IL-4, (e) IL-5 and (f) IL-13 where GAPDH was taken as internal control. The data from at least three independent experiments are expressed as the mean $\pm$ SEM. (* $p < 0.05$).

1995). Mast cell protease-1 (MCPT-1) is primarily expressed in intestinal mucosal mast cells and its expression is enhanced in gastrointestinal inflammation and allergic conditions as population of mucosal mast cells (MMCs) expands rapidly both in the mucosa and within the epithelium (Ferguson et al., 1987). Thymic stromal lymphopoietin (TSLP) is a cytokine required for normal CD4⁺ T cell-mediated inflammatory response. TSLP acts directly on naive T-cells but not on memory CD4⁺ T cells and promotes their proliferation and differentiation in response to antigen (Al-Shami et al., 2005). In the present study, the enhanced serum levels of MCP-1, MCPT-1 and TSLP was found indicating that CP 26 kDa protein has the potential to activate naive T-cells that results into their proliferation and differentiation into Th2 cells.

In addition, we examined the MPO level in lungs and intestine of purified protein treated mice as the MPO is abundantly expressed in neutrophils and its secretion is upregulated in case of allergic condition (Bradley et al., 1982). Significantly increased levels of MPO in these organs indicate the infiltration of neutrophils due to treatment of CP purified protein.

Furthermore, cross linking of allergens to IgE bound to FcεRI receptors on surface of mast cells results into mast cell activation, degranulation and release of chemical mediators like histamine, prostaglandins, leukotrienes, proteases, chymases etc. Histamine, a bioactive amine is the main proinflammatory mediator that is responsible for at least half of the signs and symptoms of the allergic reaction (White, 1990). Prostaglandin D2 is also an important mediator released from activated mast cells, accounts for a wide range of biological activities including bronchoconstriction, vasodilatation and increased vascular permeability (Nagoshi et al., 1998). Cysteinyl leukotrienes (CysL) were previously regarded as slow-reacting substance of anaphylaxis that regulates many of the features of inflammation and allergy including bronchial constriction, edema, and eosinophilia (Bisgaard, 2000). The elevated serum

levels of histamine, PGD2 and CysL in CP 26 kDa protein treated mice confirm the severity of allergy.

RBL-2H3 cells are used as a pivotal tool in determining the extent of allergic reactions caused due to exposure of allergens. In current study, we passively sensitized the RBL-2H3 cells with sera of CP 26 kDa protein treated mice and then same protein was added in different concentrations. Dose dependent release of β -hexosaminidase from RBL-2H3 cells was observed. Further, enhanced levels of histamine, PGD2 and CysL in culture supernatant of RBL-2H3 cells were found when treated with CP 26 kDa protein. The release of these allergic mediators indicates the mast cells/basophils degranulation potential of this purified protein.

Additionally, histopathological changes were observed in lungs, spleen and intestine of CP 26 kDa protein treated mice. Distortion of intestinal epithelium and villi, inflammatory cells infiltration in peribronchiolar spaces of lungs, and hyperplasia in spleen indicates the sign of allergic responses. Airway hyper-responsiveness and recruitment of inflammatory cells in lungs indicated the inflammatory response as in the case of asthma (O'Byrne, 1999). Destruction of mucosal lining and inflammatory cell infiltration characteristic feature of diarrhea, commonly reported in case of allergic manifestations in the intestine. The lymphoid hyperplasia observed in spleen may be due to massive proliferation and differentiation of T lymphocytes, reflects the signs of chronic inflammation in the lymphoid organ (Koyasu, 2012). We also found infiltration and recruitment of eosinophils in intestine, lungs and spleen of purified protein treated mice representing that this protein is responsible for secretion of various chemokines that cause recruitment of eosinophils mediated allergic complications in various tissues. Furthermore, GATA-binding proteins are a subfamily of zinc finger transcription factors. GATA-3 is important in inducing the expression of a number of T-helper 2 (Th2) cell-specific cytokines such as interleukin IL-4, IL-5 and IL-13 (Zheng and Flavell, 1997). On the

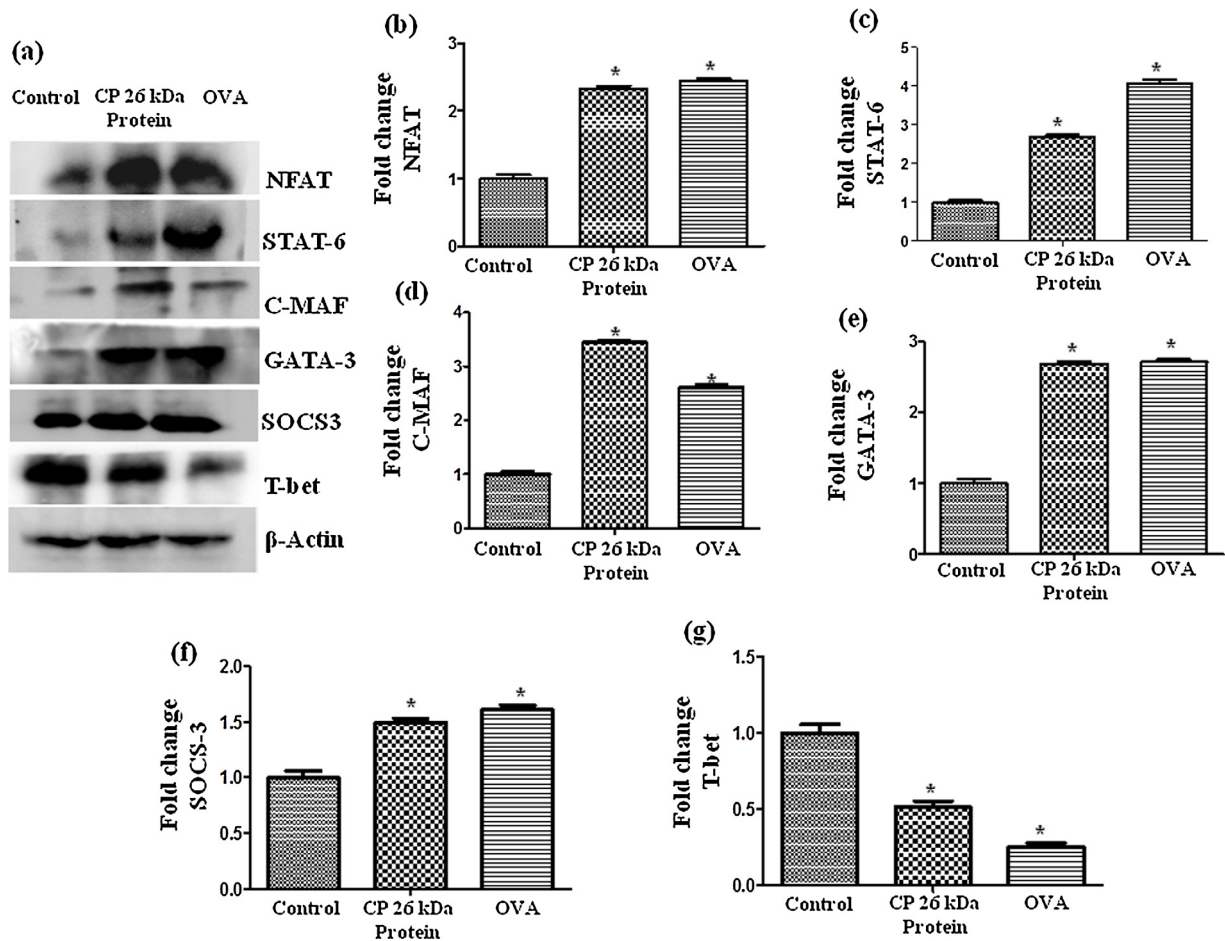


Fig. 8. (a) Western blotting for Th2 and Th1 transcription factors NFAT, STAT-6, C-MAF, GATA-3, SOCS-3 and T-bet levels in the intestine of control, CP 26 kDa protein and OVA treated groups, where β -Actin was taken as internal control. Densitometry of (b) NFAT, (c) STAT-6, (d) C-MAF, (e) GATA-3, (f) SOCS-3 and (g) T-bet. The data from at least three independent experiments are expressed as the mean $\pm$ SEM. (* $p < 0.05$).

other hand, T-bet, a Th1 transcription factor is responsible for inhibition of Th2 mediated allergic reactions and expression of T-bet is downregulated in allergic reactions and inflammation. A study revealed that antigen induced inflammatory cell infiltration in the peribronchial and perivascular areas were significantly enhanced in T-bet^{-/-} mice compared with WT mice (Fujiwara et al., 2007). In the present study, upregulated levels of GATA-3 and decreased levels of T-bet in different organs of CP 26 kDa protein treated mice were observed indicating towards the ability of purified protein to cause inflammation and allergic response in different organs of sensitized individual.

Allergic diseases are characterized by aberrant cytokine milieu and transcription factors. IL-4 is important cytokine for development of Th2 cells from naive T-cells and class switching of IgE in response to allergen (Hamelmann et al., 1999). IL-5 is a chief cytokine required for eosinophil development and differentiation (Sanderson, 1992). IL-13 plays a key role in production and class switching of IgE (Kimata et al., 1995). Th2 transcription factor GATA-3 is important for induction of allergic diseases as it is essential for gene expression of Th2 cytokines including IL-4, IL-5 and IL-13 through chromatin remodelling (Barnes, 2008). SOCS-3 is a member of suppressor of cytokine signaling family of transcription factors that regulates Th2 mediated allergic response (Seki et al., 2003). STAT-6 is critical for Th2 cells differentiation and pathogenesis of allergic inflammation as it is essential for IL-4 and IL-13 signaling (Emson et al., 1998). C-MAF, a member of AP-1 family of

basic region/leucine zipper family of transcription factors, is chiefly expressed in Th2 cells and binds to a consensus site in the proximal IL-4 promoter causes the transcription and translation of IL-4 (Kim et al., 1999). NFAT family of transcription factors are expressed in Th2 cells and in a variety of immune cells including mast cells, B-cells, eosinophils and NK cells (Rao et al., 1997). In contrast to these Th2 transcription factors, T-bet is important for the initiation of IFN- γ transcription and also inhibition of Th2 specific transcription factor GATA-3 (Usui et al., 2006). In our study, we observed induced Th2 cytokines/Th2 transcription factors and down regulated Th1 transcription factor at both mRNA and protein level in intestine of CP 26 kDa protein treated mice suggesting that immune response is skewed towards Th2 response rather than Th1 due to treatment of CP 26 kDa protein.

We further analyzed the mechanism behind the CP 26 kDa protein induced allergic reactions in sensitized animals. We have extracted the intestinal protein from control and purified protein treated mice and analyzed the levels of some protein kinases and adaptor molecules that play an important role in mast cell activation and degranulation. Fc ϵ RI is a high affinity receptor for IgE present on the surface of mast cells and basophils that are chief cells in induction of allergic reaction by releasing allergic mediators (Kumar et al., 2012). The release of these mediators is a complex process and includes a number of protein kinases, adaptor molecules, transcription factors and cytokines. Cross linking of allergen with IgE bound to the Fc ϵ RI receptor on the surface of

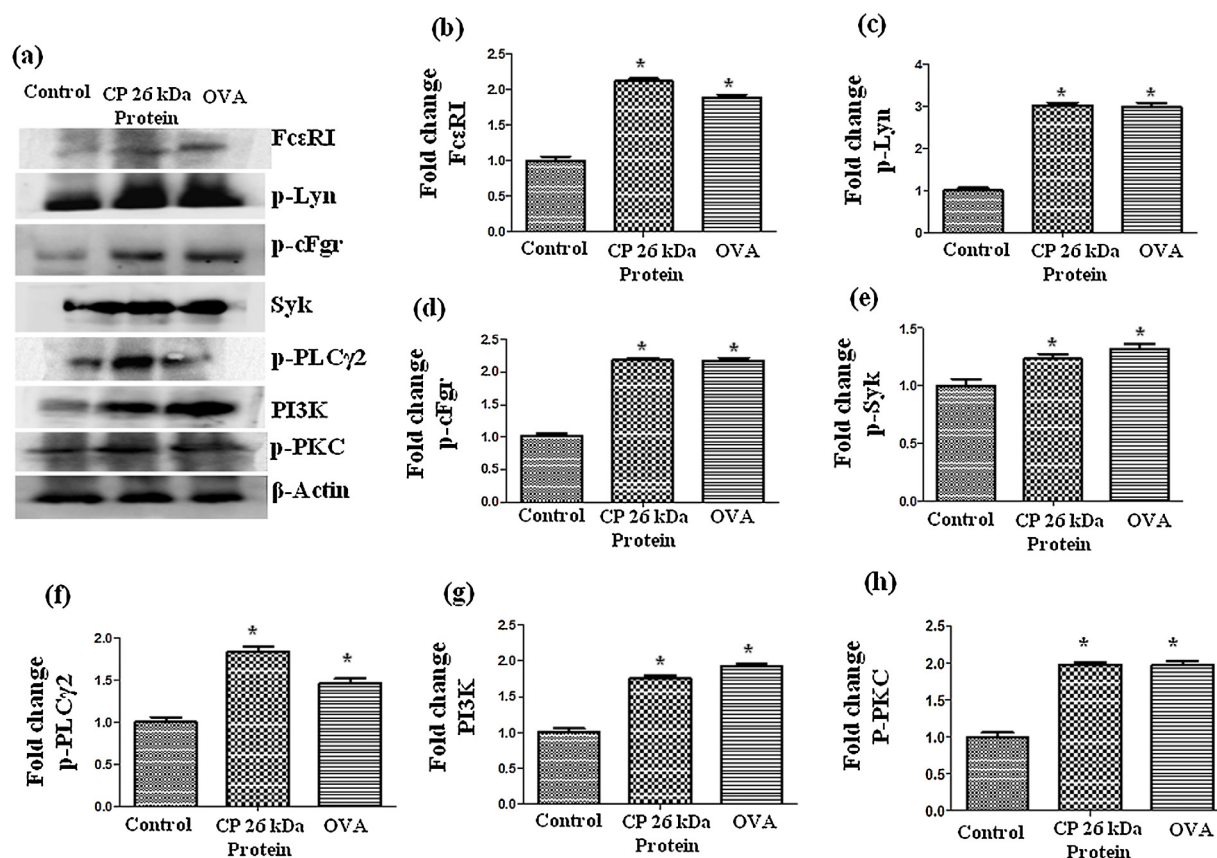


Fig. 9. (a) Western blotting for analysis of levels of signaling proteins of mast cells FcεRI receptor, p-Lyn, p-cFgr, p-Syk, p-PLCγ2, PI3K and p-PKC in the intestine of control, CP 26 kDa protein and OVA treated groups, where β-Actin was taken as internal control. Densitometry of (b) FcεRI, (c) p-Lyn, (d) p-cFgr, (e) p-Syk, (f) p-PLCγ2 (g) PI3K and (h) p-PKC. The data are expressed as the mean ± SEM. (*p < 0.05).

mast cells results into activation of Src family of protein tyrosine kinases including Lyn. Some other Src kinases like c Fgr also play an important role in activation of mast cells (Lee et al., 2011). These Src kinases phosphorylate and activate another family of protein kinases- Syk kinase that further activates some adaptor molecules and phosphorylates PLCγ2 which causes breakdown of PIP2 into IP3 and DAG and results into increase in the intracellular Ca⁺⁺ concentration (IP3) and activation of Protein kinase C (DAG). Protein kinase C is activated in its phosphorylated form and is responsible for transcription and translation of various genes including cytokine genes. In our study, we have found elevated levels of FcεRI receptor, Lyn and c-Fgr kinases (members of Src family kinases), Syk kinase which is downstream to Src kinases, p-PLCγ2, and pPKC suggesting that CP 26 kDa protein has the potential to crosslink with IgE and leads to mast cell activation that ultimately results into Th2 response and allergic manifestations.

5. Conclusion

In summary, the present study showed CP 26 kDa protein as a causative factor for the allergy in CP sensitive patients. The allergenic potential of CP 26 kDa protein is confirmed by our results that showed increased levels of IgE antibody, histamine, PGD2, CysL levels in CP 26 kDa protein treated mice as compared to control. The levels of MCP-1, MCPT-1, TSLP and MPO were also found to be increased in CP 26 kDa protein treated group. Th2 cytokines and transcription factors including IL-4, IL-5, IL-13, GATA-3, SOCS-3, STAT-6, NFAT and C-MAF were also up regulated at mRNA and protein levels but the expression level of T-bet (a Th1 transcrip-

tion factor) was decreased in the purified protein treated group. Further, purified CP 26 kDa protein also accounts for mast cell activation and degranulation via Src family kinases. Taken together, our experimental evidences indicated the potential allergenicity of CP 26 kDa protein that may be responsible for provoking allergic manifestations in the sensitized individual. The data presented in this study will be helpful for production of genetically modified hypoallergenic chickpea varieties and for developing therapeutic approaches to save susceptible population.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2016.05.002>.

References

- Al-Shami, A., Spolski, R., Kelly, J., Keane-Myers, A., Leonard, W.J., 2005. A role for TSLP in the development of inflammation in an asthma model. *J. Exp. Med.* 202 (6), 829–839.
- Barnes, P.J., 2008. Role of GATA-3 in allergic diseases. *Curr. Mol. Med.* 8 (5), 330–334.
- Bisgaard, H., 2000. Role of leukotrienes in asthma pathophysiology. *Pediatr. Pulmonol.* 30 (2), 166–176.
- Boyce, J.A., Assa'ad, A., Burks, A.W., Jones, S.M., Sampson, H.A., Wood, R.A., et al., 2010. Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. *J. Allergy Clin. Immunol.* 126 (Suppl. 6), S1–58.
- Bradley, P.P., Christensen, R.D., Rothstein, G., 1982. Cellular and extracellular myeloperoxidase in pyogenic inflammation. *Blood* 60 (3), 618–622.
- Cardoso, C.R., Provinciatto, P.R., Godoi, D.F., Ferreira, B.R., Teixeira, G., Rossi, M.A., et al., 2009. IL-4 regulates susceptibility to intestinal inflammation in murine food allergy. *Am. J. Physiol. Gastrointest. Liver Physiol.* 296 (3), G593–G600.
- Conti, P., Boucher, W., Letourneau, R., Feliciani, C., Reale, M., Barbacane, R.C., et al., 1995. Monocyte chemoattractant protein-1 provokes mast cell aggregation and [3H]5HT release. *Immunology* 86 (3), 434–440.
- Diesner, S.C., Knittelfelder, R., Krishnamurthy, D., Pali-Schöll, I., Gajdzik, L., Jensen-Jarolim, E., et al., 2008. Dose-dependent food allergy induction against ovalbumin under acid-suppression: a murine food allergy model. *Immunol. Lett.* 121 (1), 45–51.
- Emson, C.L., Bell, S.E., Jones, A., Wisden, W., McKenzie, A.N., 1998. Interleukin (IL)-4-independent induction of immunoglobulin (Ig)E, and perturbation of T cell development in transgenic mice expressing IL-13. *J. Exp. Med.* 188 (2), 399–404.
- Ferguson, A., Cummins, A.G., Munro, G.H., Gibson, S., Miller, H.R., 1987. Roles of mucosal mast cells in intestinal cell-mediated immunity. *Ann. Allergy* 59 (5 Pt 2), 40–43.
- Fujiwara, M., Hirose, K., Kagami, S., Takatori, H., Wakashin, H., Tamachi, T., et al., 2007. T-bet inhibits both TH2 cell-mediated eosinophil recruitment and TH17 cell-mediated neutrophil recruitment into the airways. *J. Allergy Clin. Immunol.* 119 (3), 662–670.
- Gopalan, C., Sastri, B.V.R., Balasubramanian, S.C., 1989. Nutritive Value of Indian Foods. National Institute of Nutrition. Indian Council for Medical Research, Hyderabad.
- Gunn, M.D., Nelken, N.A., Liao, X., Williams, L.T., 1997. Monocyte chemoattractant protein-1 is sufficient for the chemotaxis of monocytes and lymphocytes in transgenic mice but requires an additional stimulus for inflammatory activation. *J. Immunol.* 158 (1), 376–383.
- Hamelmann, E., Wahn, U., Gelfand, E.W., 1999. Role of the Th2 cytokines in the development of allergen-induced airway inflammation and hyperresponsiveness. *Int. Arch. Allergy Immunol.* 118 (2–4), 90–94.
- Helm, R.M., 2002. Food allergy animal models: an overview. *Ann. N Y Acad. Sci.* 964, 139–150.
- Kim, J.I., Ho, I.C., Grusby, M.J., Glimcher, L.H., 1999. The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. *Immunity* 10 (6), 745–751.
- Kimata, H., Fujimoto, M., Furusho, K., 1995. Involvement of interleukin (IL)-13, but not IL-4, in spontaneous IgE and IgG4 production in nephrotic syndrome. *Eur. J. Immunol.* 25 (6), 1497–1501.
- Koyasu, S., 2012. Natural helper cells and TH2-type innate immunity. *Cornea* 31 (Suppl. 1), S20–4.
- Kumar, S., Verma, A.K., Das, M., Dwivedi, P.D., 2012. Molecular mechanisms of IgE mediated food allergy. *Int. Immunopharmacol.* 13 (4), 432–439.
- Kumar, S., Sharma, A., Neelabh, Singh, G., Verma, A.K., Roy, R., et al., 2014. Allergic responses of green gram (*Vigna radiata* L: Millsp) proteins can be vitiated by induction of oral tolerance due to single acute dose in BALB/c mice. *Food Res. Int.* 57, 130–141.
- Lee, J.H., Kim, J.W., Kim do, K., Kim, H.S., Park, H.J., Park, D.K., et al., 2011. The Src family kinase Fgr is critical for activation of mast cells and IgE-mediated anaphylaxis in mice. *J. Immunol.* 187 (4), 1807–1815.
- Li, M.S., Sun, L., Satoh, T., Fisher, L.M., Spry, C.J., 1995. Human eosinophil major basic protein, a mediator of allergic inflammation, is expressed by alternative splicing from two promoters. *Biochem. J.* 305 (Pt. 3), 921–927.
- Li, X.M., Serebrisky, D., Lee, S.Y., Huang, C.K., Bardina, L., Schofield, B.H., et al., 2000. A murine model of peanut anaphylaxis: T- and B-cell responses to a major peanut allergen mimic human responses. *J. Allergy Clin. Immunol.* 106 (1 Pt. 1), 150–158.
- Martinez, S.I.M., Ibáñez Sandín, M.D., Fernandez-Caldas, E., Marañon Lizana, F., Rosales Fletes, M.J., Laso Borrego, M.T., 2000. Specific IgE levels to Cicer arietinum (chick pea) in tolerant and non-tolerant children, evaluation of boiled and raw extracts. *Int. Arch. Allergy Immunol.* 121 (2), 137–143.
- McKenzie, G.J., Bancroft, A., Grecis, R.K., McKenzie, A.N., 1998. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr. Biol.* 8 (6), 339–342.
- Misra, A., Prasad, R., Das, M., Dwivedi, P.D., 2009. Probing novel allergenic proteins of commonly consumed legumes. *Immunopharmacol. Immunotoxicol.* 31 (2), 186–194.
- Misra, A., Kumar, R., Mishra, V., Chaudhari, B.P., Tripathi, A., Das, M., et al., 2010. Partial characterization of red gram (*Cajanus cajan* L: Millsp) polypeptides recognized by patients exhibiting rhinitis and bronchial asthma. *Food Chem. Toxicol.* 48 (10), 2725–2736.
- Misra, A., Kumar, R., Mishra, V., Chaudhari, B.P., Raisuddin, S., Das, M., et al., 2011. Potential allergens of green gram (*Vigna radiata* L. Millsp) identified as members of cupin superfamily and seed albumin. *Clin. Exp. Allergy* 41 (8), 1157–1168.
- Nagoshi, H., Uehara, Y., Kanai, F., Maeda, S., Ogura, T., Goto, A., et al., 1998. Prostaglandin D2 inhibits inducible nitric oxide synthase expression in rat vascular smooth muscle cells. *Circ. Res.* 82 (2), 204–209.
- Nipadkar, P.V., Patil, S.P., Bapat, M.M., 1997. Chickpea-induced anaphylaxis. *Allergy* 52 (1), 115–116.
- O'Byrne, P.M., 1999. Inhaled corticosteroid therapy in newly detected mild asthma. *Drugs* 58 (Suppl. 4), 17–24.
- Rao, A., Luo, C., Hogan, P.G., 1997. Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* 15, 707–747.
- Sanderson, C.J., 1992. Interleukin-5, eosinophils, and disease. *Blood* 79 (12), 3101–3109.
- Seki, Y., Inoue, H., Nagata, N., Hayashi, K., Fukuyama, S., Matsumoto, K., et al., 2003. SOCS-3 regulates onset and maintenance of T(H)2-mediated allergic responses. *Nat. Med.* 9 (8), 1047–1054.
- Sicherer, S.H., Sampson, H.A., 2010. Food allergy. *J. Allergy Clin. Immunol.* 125 (2 Suppl. 2), S116–S125.
- Špičák, V., 2010. Changing face of allergy and allergic diseases. *Alergie* 12, 83–86.
- Usui, T., Preiss, J.C., Kanno, Y., Yao, Z.J., Bream, J.H., O'Shea, J.J., et al., 2006. T-bet regulates Th1 responses through essential effects on GATA-3 function rather than on IFNG gene acetylation and transcription. *J. Exp. Med.* 203 (3), 755–766.
- Verma, A.K., Kumar, S., Tripathi, A., Chaudhari, B.P., Das, M., Dwivedi, P.D., 2012. Chickpea (*Cicer arietinum*) proteins induce allergic responses in nasobronchial allergic patients and BALB/c mice. *Toxicol. Lett.* 210 (1), 24–33.
- Verma, A.K., Kumar, S., Das, M., Dwivedi, P.D., 2013. A comprehensive review of legume allergy. *Clin. Rev. Allergy Immunol.* 45 (1), 30–46.
- White, M.V., 1990. The role of histamine in allergic diseases. *J. Allergy Clin. Immunol.* 86 (4 Pt. 2), 599–605.
- Zheng, W., Flavell, R.A., 1997. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4T cells. *Cell* 89 (4), 587–596.

Supplementary Data

S1. Chickpea crude protein extraction (CP-CPE)

Seeds were grinded and powder was defatted with n-hexane (Merck, Worli, Mumbai, India). Defatted powder was macerated in the phosphate buffered saline (20 mM Na₂HPO₄, 2mM KH₂PO₄, 5.4 mM KCl, 0.5 M NaCl, pH 7.0). The mixture was agitated overnight at 4°C, centrifuged for 30 min at the 10,000×g and supernatant was recovered, filtered through 0.45 µm syringe filter and stored as aliquots at –80 °C until used.

S2. Characterization of the purified protein by Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Purified CP 26 kDa protein was excised from Coomassie Brilliant Blue G-250-stained gel for LC-MS/MS study. The LC-MS/MS studies were carried out at The Centre for Genomic Applications (TCGA), New Delhi. In brief, the purified protein was cut into small pieces, followed by destaining and tryptic in-gel digestion (Schrimpf et al., 2005). The supernatant of the digested solution was collected and the gel pieces were extracted with 100 µl of 20 mM NH₄CO₃ for 20 minutes and this supernatant was recollected. Again gel pieces were extracted twice with 100 µl of 1% TFA in 50% acetonitrile for 20 min and supernatants were collected. All the supernatants were pooled, injected into Thermofinnigan LCQ DECA XP (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a capLC pump (Waters). The individual MS/MS spectra of tryptic fragments were searched against mass spectrometry protein sequence Data Base (MSDB) using MASCOT search engine (Perkins et al., 1999). Mass tolerance and monoisotopic values (peptide mass tolerance of ±1.2 Da and fragment mass tolerance of ±0.6 Da for MS/MS spectra) were used for searching. Carbamidomethyl was considered as fixed modification of tryptic fragments with oxidation as variable modification. Probability based MOWSE score was calculated in terms of ion score $-10 \times \log(P)$, where P is the probability and observed match was considered as a random event.

Further, bioinformatics approach was used for allergenicity prediction of purified protein using Algpred and SDAP software. Algpred predicts allergenicity of protein sequences using different approaches namely:- IgE epitope mapping, MEME/MAST motif, Support Vector Machine (SVM) based on amino acids composition, Support Vector Machine (SVM) based on dipeptide composition, Allergen Representative Peptide (ARP) method, hybrid method.

S3. IgE Immunoblot analysis

CP purified protein was resolved on 12% SDS–PAGE and electrophoretically transferred to a polyvinyl difluoride membrane (Immobilon P 0.45um, Millipore, Bangalore) using a semi-dry blotting unit (Amersham Biosciences, St Francisco, USA). The nonspecific sites were blocked with blocking buffer (3% BSA in PBS-T) and kept for 2 hr at 37 °C. Washing was done by PBS-T for 5 times. CP purified protein sensitized mice sera and SPT positive human sera were added as primary antibody (1:20) and blots were kept at 4 °C overnight. Peroxidase conjugated goat anti-mouse IgE (Southern Biotech, Birmingham, AL, USA, dilution 1:1000 in PBS-T+1% BSA) and goat anti-human IgE peroxidase conjugated (Sigma Chemical Company, dilution 1:1000 in PBS-T+1% BSA) were used as secondary antibody respectively. The blot was developed by chemiluminescence method (Pierce, Thermo Scientific Rockford, USA) and image was acquired using a gel documentation system (Syngene Bio Imaging System, Cambridge, UK).

S4. Myeloperoxidase (MPO) assay

100 mg of tissue from PBS, CP purified protein and OVA treated mice were homogenized in two volumes of ice cold buffer (0.1 M NaCl, 20 mM NaPO₄, 15 mM Na EDTA, pH 4.7) and centrifuged at 600 g for 15 min. The pellet was subjected to a hypotonic lysis in 900 µl of 0.2% NaCl solution. After 30 seconds, an equal volume of a solution containing 1.6% NaCl and 5% glucose was added. Following centrifugation, the pellet was re-suspended in 50 mM NaPO₄ buffer (pH 5.4) containing 0.5% hexadecyl trimethyl ammonium bromide (Sigma

chemicals, St. Louis, MO, USA) and re-homogenized. The homogenate was then frozen, thawed 3 times and centrifuged at 3,000 g for 15 min at 4 °C. The MPO activity in the supernatant was assayed by measuring the change in absorbance at 450 nm using tetramethylbenzidine substrate (TMB reagent set, BD Biosciences, San Jose, CA, USA). The results were reported as change in absorbance at 450 nm/ gram of tissue.

S5. Semi quantitative polymerase chain reaction (PCR)

Total RNA from intestine was isolated with RNeasy[®] Lysis Reagent (Qiagen, Crawley, UK) according to manufacturers' instructions. The integrity of RNA was determined by running on 1.2% agarose gel prior to further processing. The RT-PCR was performed by commercially available RT-PCR kit (Qiagen, Netherland). The GAPDH gene was chosen as an endogenous control. The program of thermal cycler was as follows; reverse transcription at 50 °C for 30 min and subsequent 40 cycles of 94 °C denaturation for 30 s, 55 °C annealing for 30 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. Further, 8 µl of PCR product was run on 2% agarose gel containing ethidium bromide. Densitometry of each band was done by the Gene tools software (Syngene, Cambridge, UK).

S6. Western blots for transcription factors

Intestinal proteins of control, CP 26 kDa protein and OVA treated mice were extracted in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na₃VO₄, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 mg/mL aprotinin, 10 mg/mL leupeptin, pH 7.4). Intestinal proteins were resolved on 12% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon P 0.45µm, Millipore, Bangalore) using a semi-dry blotting unit (Amersham Biosciences, St Francisco, USA). The nonspecific sites were blocked with blocking buffer (3% BSA in PBS-T) and kept for 2 hr at 37 °C. Washing was done by PBS-T for 5 times. The goat anti-mouse NFAT, STAT6, C-MAF, SOCS3, GATA-3, T-bet antibodies (dilution 1:200 in PBS-T + 3% BSA)

were used as primary antibodies, respectively. The rabbit anti goat IgG peroxidase conjugate was used as secondary antibody. The β -actin (Santa Cruz biotechnology, CA, USA) was used as an endogenous control. Images were captured using Syngene gel documentation system equipped with a CCD camera (Syngene, Cambridge, UK). Densitometry of each band was performed by the Gene tools software (Syngene, Cambridge, UK).

Supplementary table 1: LC–MS/MS analysis of CP 26 kDa protein

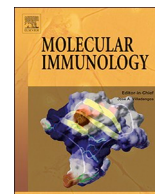
Protein sequence	Sequence similarity with proteins and their accession	Score	Mol. wt. (kDa)
1TKTGYINAAF RSSRNNEAYL FINDKYVLLD YAPGTSNDKV LYGPSFVRDG 51YKSLAKTIFG TYGIDCSFDT EYNEAFIFYE NFCARIDYAP HSDKDKIISG 101PKKIADMFPF FKGTVFENGI DAAFRSTK GK EVYLFKGD KY ARIDYLTNRL 151VQNKSISDTG FPCLRGTIFE AGMDSAFASH KTNEAYL FKG EYYARINFTP 201GSTNDIMGGV KKTLDYWPSL RGIIPLE	Chain A, Crystal Structure Of A Plant Albumin From Cicer arietinum gi 339961381	201	25.8, pI-8.24

Supplementary table 2: Allergenicity prediction of purified 26 kDa chickpea protein using bioinformatics approach

Allergenicity prediction according to Algpred software					
S.N.	Prediction criteria			Purified protein 26 kDa	
1	Mapping of IgE epitope			Non allergen	
2	MEME/MAST motif			Non allergen	
3	SVM module based on amino acid composition			Allergen	
4	SVM module based on dipeptide composition			Allergen	
5	Blast search on allergen representative peptides (ARPs)			Non allergen	
6	Hybrid Approach (SVMc+IgE epitope+ARPs BLAST+MAST)			Allergen	
Allergenicity prediction according to SDAP software					
S.N.	Allergen	Sequence Link in SwissProt/NCBI/PIR	Sequence Length	Sequence identity	E score
1	Vig r 4.0101	Q43680	272	55.07	2.7e-50

Where: MEME= Maximum Em for Motif Elicitation; MAST= Motif Alignment and Search Tool; SVM= Support Vector Machine-based; ARPs= Allergen Representative Peptides; BLAST= Basic Local Alignment Search Tool.

SDAP = Structural Database of Allergenic Proteins. The FASTA alignments between the query sequence and all SDAP allergens have an E score higher than 0.010000. Alignment made with FASTA 3.45



Corrigendum

Corrigendum to “Purification, characterization and allergenicity assessment of 26kDa protein, a major allergen from *Cicer arietinum*” [Mol. Immunol. 74 (2016) 113–124]



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The authors regret to inform that there was an inadvertent error in figure 5(b) which has now been replaced with the correct image. The entire panel of this figure is appended below including the correct image of figure 5(b). However, this error will not affect the result and scientific conclusion of the manuscript.

The authors would like to apologise for any inconvenience caused. Figure 5(b). The immunohistochemical analysis depicting EMBP expression in lungs of OVA treated group has been changed.

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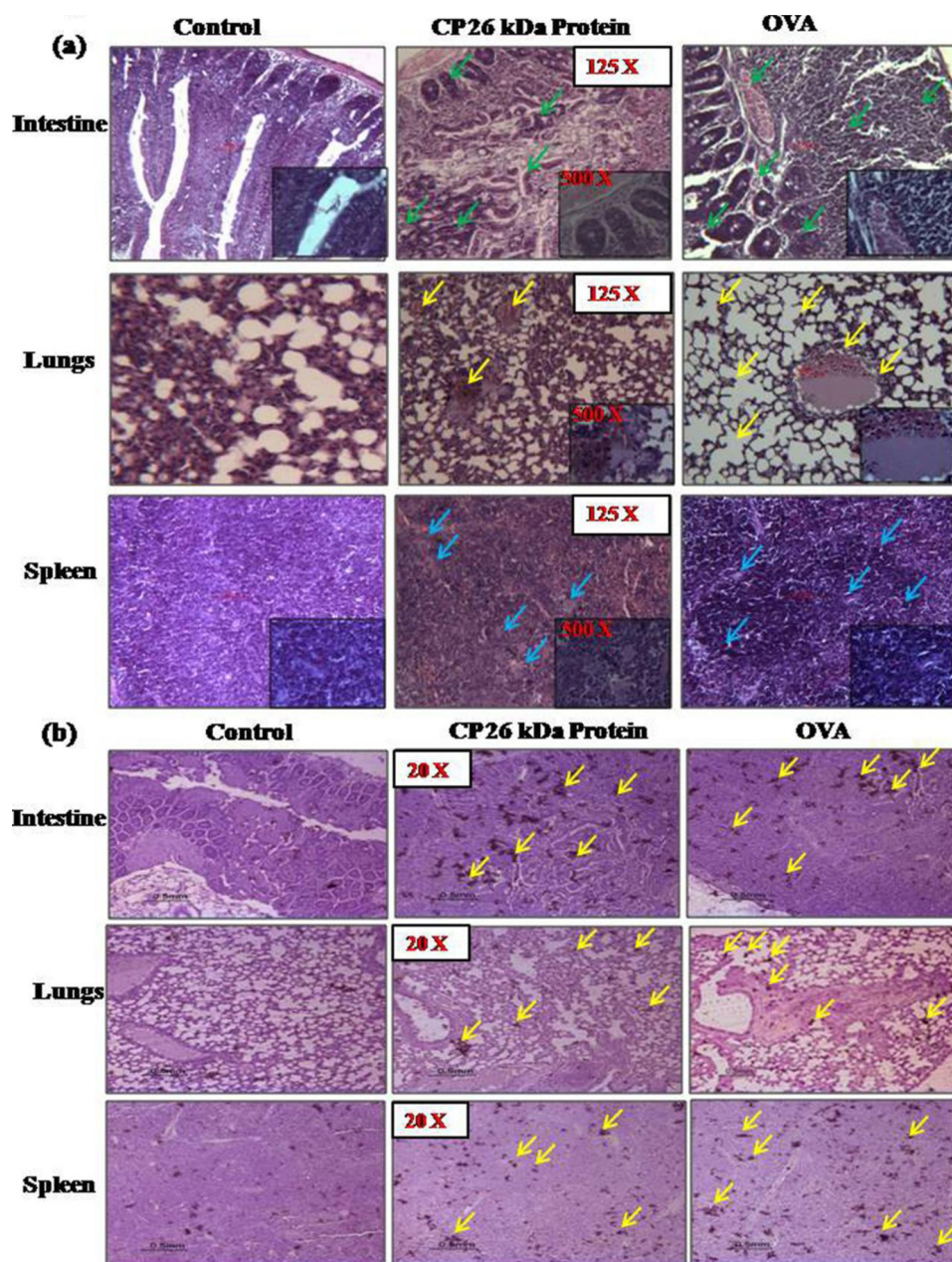


Fig. 5: (a) Histopathological analysis of intestine, lungs and spleen of control, CP 26 kDa protein and OVA treated mice groups. Intestines of treated groups showed exfoliation of villi with infiltration of inflammatory cells (indicated with green arrow), lungs showed hyperplasia of bronchial lining epithelium and thickening of alveolar septa (indicated with yellow arrow), spleen depicted megakaryocytes and lymphoid hyperplasia (indicated with blue arrow). Histological images

has been taken at 125X and 500X for better clarity (b) Immunohistochemical analysis for eosinophils in intestine, lungs and spleen of control, CP 26 kDa protein and OVA treated groups. Significant increase in eosinophil count was observed in intestine, lungs and spleen of CP 26 kDa protein and OVA treated groups when compared to control.