



Glycation of clinically relevant chickpea allergen attenuates its allergic immune response in Balb/c mice



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ABSTRACT

Glycation of food allergens may alter their immunological behaviour. We sought to investigate the impact of glycation on the allergenicity of a food protein. Herein, a chickpea protein (≈ 26 kDa) was purified and characterized as lectin. Further, glycation of this purified protein was carried out. Thereafter, allergic behaviour of this glycated protein was compared with its native form, using various allergic parameters in Balb/c mice.

The reduced allergenicity of glycated protein was observed as lesser allergic phenotypes, reduced serum immunoglobulins and allergic mediators, lower mast cells and eosinophil counts, lower protein expressions of Th2 cytokines and associated transcription factors. In addition, more Th1 and less Th2 cytokine production in exposed splenocyte, were evident in the glycated protein treated mice as compared to its native protein treatment. Thus, glycation of the chickpea allergen attenuated the sensitizing potential and allergic responses in Balb/c mice significantly and could also be clinically beneficial.

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1. Introduction

In the past few decades, consumption of highly processed foods has dramatically increased. Among the different processing methods, legumes mostly undergo thermal processing prior to their consumption. Any food processing method, if employed, may influence its allergenicity in several ways. Available food processing

methods can either increase or decrease, the allergenicity of an isolated food protein, but the overall effect on a food allergen associated with food matrix cannot be predicted without performing it practically (Gupta et al., 2016; Pastorello, 2004; Wal, 2003). The impact of any type of food processing method on the allergenicity depends upon the structure of responsible protein, as well as its biochemical composition and the nature/condition of the employed processing method used. Apart from this, interactions of food allergen with other food matrix constituents may occur and subsequently play a significant role in the overall allergenicity modulation (Davis & Williams, 1998). In this regard, thermal

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processing is the most extensively applied method to attenuate the allergic behaviour of food allergens that did not exert a positive effect on all allergens. Usually, heating to temperatures in the range between 70–80 °C can suppress the allergenicity by denaturation of responsible protein (Davis & Williams, 1998). Although, many thermo-stable food allergens, such as Gly m 4, Bet v 1 and hazelnut allergen, can not be altered by thermal processing even at high temperatures (Mondoulet et al., 2005). In certain cases, an enhancement in the allergenicity by neo-allergens formation has also been found after thermal processing (Gupta et al., 2016; Mondoulet et al., 2005). In addition, the flavour, taste, and nutritional value of certain foods may be compromised after thermal treatment at high-temperature.

However, non conventional thermal processing methods are found to be better as these methods can preserve the natural taste during the retaining of nutritional value and better organoleptic properties of the food. Although, many limitations remain in their application to overcome the food allergenicity. For instance, due to poor penetration of ultrasound treatment, pulsed UV light and irradiation destroy only those food allergens that are found on the food surface. Furthermore, the genetic modification can produce genetically modified food/crops without the allergen by silencing the allergenic genes, but application of this procedure requires crossing many hurdles. Several hypoallergenic foods are being marketed that are mostly manufactured by the enzymatic hydrolysis method, but this method could limit their organoleptic properties and reveal a negative impact on the structural composition of the foods (Houska, Setinova, & Kucera, 2011; Shriver & Yang, 2011). In recent times, high-pressure-processed (HPP) foods have become popular due to their excellent organoleptic quality and retaining of nutritional value. The HPP may affect allergenicity by altering the structure and properties of food allergens. Regarding food protein, the impact of high pressure is associated to the breaking of non-covalent interaction; consequently it affects only secondary, tertiary and quaternary structure of proteins that are maintained by non-covalent bonds (Yaldagard, Mortazavi, & Tabatabaie, 2008). Therefore, high-pressure treatment has great potential in reducing the food allergenicity without any adverse effect on the integrity of primary structure of proteins maintained by covalent bonds but, in this case, linear epitopes may retain allergenicity. Apart from this, HPP may affect the microstructure of low moisture containing foods. Therefore, the development of other potential processing techniques are necessary in order to minimize the risk of allergy, as well as preserving the nutritional value of food.

In recent years, many studies have begun to explore the effects of non enzymatic glycation on the allergenicity of food allergens, which are explained by different mechanisms, such as induction of protein conformational changes or modifications of allergen epitopes. During certain types of thermal processing, generally a non enzymatic glycation reaction may occur, which is known as the Maillard Reaction (MR), causing glycation of food proteins/allergens. Glycation of food allergens via MR occurs due to the reaction between protein having free amino groups and the free carbonyl groups of participating reducing sugar. The influence of MR on allergenicity of several allergens from leguminous as well as non-leguminous foods, including soy, peanut, egg, potato, milk, buckwheat, almond and shellfish has been reported (Gupta et al., 2016). The data from these studies clearly demonstrate that glycation of allergens via MR can significantly alter the immunogenicity, as well as allergenicity by promoting or suppressing the allergic responses caused by certain food allergens in the terms of IgE binding property. Thus, apart from the adverse health effects, it can also prove to be a safe and efficacious arsenal to combat the food allergy. The existing knowledge regarding the MR in food allergy is based upon IgE binding property and allergic mediators release assay.

Although, the human diet has a great diversification, only a limited number of foods are responsible for the majority of food allergies (Sampson, 2004). Currently known plant food allergens may be categorized into about 30 protein families (Hauser et al., 2008). Most of the plant food allergens belong to few protein families, i. e. cupin (7S & 11S seed storage proteins) and the prolamins (2S albumin, LTP, trypsin inhibitors, prolamins storage protein). Chickpea (CP) being one of these possesses 2S albumin ($\approx$ 20 kDa and $\approx$ 26 kDa) as a major constituent of allergic proteins (Gupta et al., 2017), suggesting that 2S albumin of chickpea may play a pivotal role in the induction of CP allergy. Chickpea or Garbanzo bean (*Cicer arietinum* L.) is a protein rich legume that belongs to the *Fabeaceae* family. Chickpea seeds contain seven IgE-binding proteins with mol. Wt. of 20, 26, 35, 45, 55, 70 and 95 kDa that may induce the allergic manifestation in sensitive individuals as well as in Balb/c mice (Verma et al., 2012).

During thermal processing, MR influence on the nutritional value of chickpea has been studied (Rachwat, Nebesny, & Budryn, 2013), but, impact of MR on the allergenicity of chickpea allergen is still unknown. Therefore, the impact of non enzymatic glycation on the allergenicity of chickpea allergen should be taken into consideration. Hence, this major allergenic protein (26 kDa) was of interest in the present study.

Considering the above facts, attempts were made to purify a clinically relevant allergen (26 kDa) from chickpea that belongs to the plant albumin family. Furthermore, non-enzymatic glycation as well as characterization of native and glycated chickpea allergen was carried out. Thereafter, Balb/c mice were administered with vehicle control, native purified and glycated purified allergic protein, respectively in separate groups and comparative allergic responses of glycated vs non-glycated chickpea protein was evaluated.

2. Materials and methods

2.1. 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. Chickpea crude protein extract (CP-CPE) was prepared as previously described (Verma et al., 2012). In brief, seeds were ground in a blender and the resultant powder was de-fatted with n-hexane (Merck, Worli, Mumbai, India), and then macerated in phosphate-buffered saline (PBS, pH 7.0). The mixture was then agitated overnight at 4 °C, then centrifuged for 30 min at 10000 g. The resulting supernatant was recovered, filtered through a 0.45 μ m filter and stored (in aliquots) in a –80 °C freezer until used.

2.2. Purification and characterization of 26 kDa protein having IgE binding property

The purification of chickpea albumin (26 kDa) was carried out as previously described (Kumar, Sharma, Das, Jain, & Dwivedi, 2014). Purification of the IgE binding protein from CP-CPE was carried out using a combination of fractionation by amicon centrifuge tubes (Millipore, USA), anion exchange chromatography (GE healthcare, Little Chalfont, Buckinghamshire, United Kingdom), IgE immunoblotting and high pressure liquid chromatography (HPLC) (Water LC module equipped with a dual pump model 515, rheodyne injector with 200 μ l loop and a tunable absorbance detector model 2489) techniques. The CP-CPE was fractionated by using amicon centrifuge tubes, with 100, 50 and 30 kDa cut off values. Further, 12% SDS-PAGE was run to observe the proteins in different fractions. The fraction having major proteins around 30 kDa was selected for anion exchange chromatography. Tris (pH 8.0) and

Tris-NaCl (pH 8.0) were used to make a gradient for elution of different fractions. Further, the eluted fractions were run on 12% SDS-PAGE and the fractions containing proteins around 26 kDa were desalted and concentrated by using dialysis tubing (Sigma, USA). These fractions were further run on 12% SDS-PAGE and transferred onto the PVDF membrane (Millipore, USA) and further IgE immunoblotting using the pooled sera of chickpea sensitized mice was carried out. We next, selected the fractions showing IgE binding potential to the pooled sera of CP sensitized mice for further purification using HPLC. In HPLC, the elution of fractions was monitored at 280 nm. The semi preparative column was equilibrated by water containing 0.1% Trifluoroacetic acid (Buffer A) at 1 ml/min. Further, a nonlinear gradient elution using 0.1% Trifluoroacetic acid in acetonitrile (Buffer B) in Buffer A, with 5% Buffer B at time (T) = 5 min; 70% at T = 27 min, 80% B at T = 60 min and 100% Buffer B at T = 69 min. Further, different elutes were run of 12% SDS-PAGE. We further characterized the IgE binding protein of chickpea using LC-MS/MS as per the earlier described methods (Kumar, Sharma, Das et al., 2014) as named N-CPA was taken for further studies.

2.3. Glyco-conjugate (G-CPA) preparation

Maillard reaction as described by Usui et al. (2003) was carried out. Glyco-conjugate (G-CPA) preparation was carried out using monosaccharides D-glucose (Sigma; Oakville, ON, Canada). Briefly, the purified protein was mixed with D-glucose at 1:1 ratio (w/w; purified protein: D-glucose) and freeze-dried at 55 °C for 72 h at a 65% relative humidity using saturated phosphate buffer solution (PBS). As a control (N-CPA), purified protein was incubated in the same condition without glucose. The sample was extensively dialyzed with PBS buffer overnight at 4 °C to remove non conjugated saccharides.

2.4. Estimation of free lysine residue

The estimation of free lysine residue content was performed by previously described method (Ansari, Moinuddin, & Ali, 2009). To estimate the free lysine residues, 100 µg/ml of C-CPA and G-CPA were dissolved in 0.1 M sodium bicarbonate (pH-8.5) buffer. To 100 µl of protein solution, 50 µl of diluted TNBSA dye was added and mixed well. The mixture was incubated at 37 °C for 2 h. After incubation, 0.5 ml of 10% SDS and 0.25 ml of 1 N HCl were added to stop and stabilize the reaction. The quantity of free lysine present in the protein samples was estimated from the mean of three independent readings of absorption spectra at 420 nm with molar extinction coefficient of $19200 \text{ cm}^{-1} \text{ mol}^{-1}$.

2.5. Circular Dichroism (CD) and Fourier Transform-Infrared (FT-IR) spectroscopy

Protein's secondary structure elements were determined by CD as well as FT-IR spectroscopy. The CD spectra of native and glycated samples were recorded and scanned four times in the range of 190 to 260 nm using a Jasco J 600 spectropolarimeter (Easton, MD), whereas FT-IR measurements were carried on Shimadzu FT-IR spectrophotometer (8201-PC) in the spectral range of 700–4000 cm^{-1} .

2.6. LC-MS analysis of Maillard reaction product

Native and glycated chickpea albumin samples were analyzed by liquid chromatography mass spectrometry (LC-MS). Prior to loading, all samples were hydrolyzed as described in a previously established protocol (Arif, Ashraf, Ahmad, Arif, & Alam, 2012). Glycated protein sample was hydrolyzed using 0.1 N HCl. The hydro-

lyzed samples were filtered through 0.5 µm filter followed by reverse phase separation via Agilent 1100 series Acquity UPLC system coupled with C-18 analytical column (2–250 nm). The conditions for chromatography was 0.4% acetic acid (Solvent A, 20%), 0.2% formic acid in acetonitrile (Solvent B, 80%). The Flow rate of 0.5 ml/min was used to flow into the LC-MS system. The mass spectrometry analysis was then done on Micromass Quattro Ultima Triple Quadrupole mass spectrometer. The system was operated in positive ion mode and with a full scan range of 0–400 m/z range. Glycated protein sample was analyzed using carboxyl methyl lysine (CML) standard.

2.7. 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, Lucknow, India (Ref: IEC No. 2938/R. Cell-11). The animal study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of CSIR-IITR (Ref No- ITRC/IAEC/11/2015), Lucknow, India.

2.8. Screening of chickpea sensitive patients

Skin prick tests (SPT) were performed with the allergen kit (Alicit India Private Limited) on allergic patients (n = 100) and normal, healthy individuals (n = 10) at the Department of Respiratory Medicine, King George Medical University (KGMU), Lucknow. Weal diameter equal to positive control or more was 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 were considered.

2.9. Immunoblot analysis

To detect the IgE binding capacity of purified chickpea protein (26 kDa), IgE immunoblotting was performed using the chickpea sensitive patient's sera (Verma et al., 2012). In brief, protein was resolved on 12% SDS-PAGE and electrophoretically transferred onto PVDF membrane with pore size 0.45 µm (Immobilon-P, Millipore Co., Bedford, MA, USA) for 2 h at 0.8 mA/cm² at room temperature with a semi dry blotting unit (Amersham Biosciences, San Francisco, CA, USA). The membrane was cut into strip and blocked with 5% defatted milk in PBST buffer (Tris-HCl 20 mM, 500 mM NaCl of pH 7.4 containing 0.1% Tween-20) after termination of the electrophoretic transfer and kept overnight at 4 °C. After three washes (5 min each) with PBST, the strip was incubated with sera of chickpea allergic individuals as well as sensitized mice for overnight at 4 °C (dilution 1:10 in PBST + 3% BSA). After 3 washes again (5 min each) with PBST, the strip was then incubated for 6 h at room temperature with goat anti-human IgE peroxidase conjugate (Sigma Chemical Company, dilution 1:1000 in PBST + 3% BSA). After two additional washes (5 min each), the IgE binding proteins were visualized with 3,3'-Diaminobenzidine tetrahydrochloride, a substrate for HRP, which yields a coloured deposit.

2.10. Animal sensitization protocol

Mice were sensitized with PBS, N-CPA and G-CPA according to the earlier described method (Verma et al., 2012). The experimental mice were randomly divided into three groups (n = 15/group) and treated via intraperitoneally. Group 1 received 100 µl of phosphate-buffered saline (PBS) as a control, group 2 was administered purified 100 µg in 100 µl CP albumin and group 3 received 100 µg in 100 µl glycated CP albumin once a week. Blood samples were collected from the retro-orbital sinus to measure serum IgE and IgG1 antibodies on day 59. On day 60, the experimental mice

were challenged i.p. with 1 ml of 5 mg/ml purified CP albumin protein. Tissue and blood samples were collected for further experiments.

2.11. Assessment of anaphylactic symptoms and measurement of body temperature

Systemic anaphylactic reactions in mice were scored according to Li et al. (2000) on a scale of 0 to 5. Among all the challenged mice, scoring of a subgroup (n = 5) from each group was presented. The symptoms of anaphylaxis were observed 20–30 min after challenge and scored as follows: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth, diarrhea, and reduced activity or standing still with an increased respiratory rate; 3 = wheezing, labored respiration, and cyanosis around the mouth; 4 = symptoms as in No. 3 with loss of consciousness, tremor, and/or convulsion; and 5 = mortality (death). Rectal temperature was measured before and after 20 min of the challenge using a digital rectal thermometer.

2.12. Total and specific IgE as well as IgG1 antibodies levels

Total IgE levels in sera of control, N-CPA and G-CPA treated mice were estimated using a commercially available ELISA kit (BD Bioscience, San Jose, USA). Furthermore, 26 kDa specific IgE and IgG1 levels were estimated according to the earlier described methods (Misra et al., 2011). For this, 1 µg of 26 kDa protein in 100 µl coating buffer [carbonate buffer (pH 9.6)] was coated onto wells of microtiter plates (Maxisorp; Nunc™ Immunomodule, Roskilde, Denmark) and left overnight at 4 °C. Thereafter, the wells were washed thrice with PBS-T and unspecific sites were blocked with a solution (200 µl) of 3% BSA in PBST (w/v) for 2 h. The plates were then washed and incubated with diluted sera from the control, N-CPA and G-CPA treated mice; samples of sera were diluted (using 3% BSA in PBST) 1:20 for the IgE and 1:1000 for the IgG1 analyses. All samples were run in triplicate. The plates were incubated overnight at 4 °C and then washed five times with washing buffer (PBS-T). HRP anti mouse-IgE and IgG1 solutions (each at 1:1000) were then added to dedicated wells and the plates were incubated for 2 h at 37 °C. After washing, 100 µl substrate solution (5 mg o-phenylenediamine in 10 ml substrate buffer and 10 µl H₂O₂) was added to each wells and the plates were incubated for 30 min at 37 °C in the dark. Stop solution (50 µl of 5 N H₂SO₄) was added to each well and the absorbance (OD) in each was then measured at 492 nm in an XS2 ELISA plate reader (Biotek Power Wave, Winooski, VT). Only the absolute OD values were used for comparisons among the serum samples.

2.13. Measurement of Monocyte chemotactic protein-1 (MCP-1), serum mouse mast cell protease 1 (MCPT-1) and Thymic stromal lymphopoietin (TSLP)

MCP-1, MCPT-1 and TSLP levels in the sera of all groups were measured by ELISA method using a BD OptEIA™ kit (for MCP-1: BD Biosciences, San Diego, CA, USA, for MCPT-1 and TSLP: eBioscience, San Diego, CA, USA) according to manufacturer's instructions. The results were expressed in terms of absorbance or concentration.

2.14. Measurement of PGD2 and histamine

The quantification of prostaglandin D2 (PGD2) and histamine levels in sera of mice from both the treated groups were determined using EIA kit (for histamine: SPI-BIO, Montignyle and Bretonneux, France; for PGD2: Cayman chemicals, USA) following the manufacturers' protocol.

2.15. Immunofluorescence histochemistry for Fox-P3, IL-4 and eosinophil levels

Eosinophil count, IL-4 and FoxP3 levels were analyzed in the lung and intestine of mice from N-CPA and G-CPA groups by immunofluorescence histochemistry (Kumar et al., 2013). Rabbit anti mouse eosinophils major basic protein, goat anti mouse IL-4 and Goat anti mouse FoxP3 (Santa Cruz biotechnology, Inc., USA) and Alexa flour 594 and FITC conjugated anti-rabbit/anti-goat antibody were used primary and secondary antibody, respectively. The images were captured by confocal microscope (Leica Micosystem, Germany).

2.16. Mast cells counts

Detection of mast cell in the lung and intestine of native and glycated protein treated groups was carried out using toluidine blue staining. The sections of the tissues from all groups (3–5 µm) were rehydrated in xylene, xylene + ethanol and 100, 90, 70 and 50% ethanol in a serial manner. Sections were washed with PBS and stained with toluidine blue dye (Sigma Aldrich, USA) for 30 min. Following gentle washing in the running tap water, dehydration was carried out using 50, 70, 90 and 100% ethanol, xylene + ethanol and xylene, respectively. Slides were mounted with DPX (BDH, India) and images were captured by microscope (Nikon Eclipse TE2000-S microscope).

2.17. Flow cytometry for cytokine measurement

The quantification of cytokines was carried out by flowcytometry (BD FACS Canto II) using Th1/Th2 CBA cytokines kit (BD Biosciences, San Jose) (Misra et al., 2011). Spleen cell suspension as prepared aseptically in RPMI-1640 media (Sigma Chemical Co.). Splenocytes were seeded (1*10⁶ cells/well) in triplicate and treated with 100 µg/ml of N-CPA, G-CPA and PBS, separately. The supernatants from all groups at 24, 48 and 72 h were collected after centrifugation at 100g for 5 min and stored at –80 °C until analysis. Mouse IL-4, IL-17, IL-10, IL-2 and IFN-γ cytokine levels in culture supernatants were quantified at all time points.

2.18. Real time PCR

A real time PCR analysis of IL-4 cytokine as well as its receptor (IL-4R) and transcription factors (GATA-3, FoxP3) in the intestine of all treated groups was carried out. The total RNA from the intestinal tissues of control, N-CPA and G-CPA groups was isolated using the TRI-Reagent (Invitrogen Life Technologies, Carlsbad, CA) and cDNA were prepared using high capacity cDNA reverse transcriptase kit (Applied Biosystem, Foster City, CA). The qPCR was carried out on cDNA samples using the SYBR Green system (Bio Rad, Richmond, CA). Primers used were IL-4: sense 5'-TCGGCATTTTGAAC GAGGTC-3', antisense 5'-AAAAGCCCGAAAGAGTCTC-3'; IL-4R (IL-4 receptor): sense 5'-TCCTCTCTTTCCCTCTCT-3'; antisense 5'-CGT GCTAAGGAGCATCCAAC-3'; GATA-3: sense 5'-TCTCACTCTCGAGG CAGCATGA-3' antisense 5'-GGTACCATCTCGCCGCCACAG-3'; Foxp3: sense 5'-GCTCCCGGCTGGTCTGCTC-3', antisense 5'-AGGTGGCGG GGTGGTTTCTGA-3' and GAPDH: sense 5'-TTCACCACCATGGA GAAGGC-3', antisense 5'-GGCATGGACTGTGGTCATGA-3'. Thermal cycling conditions were 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles each corresponding to 15 s at 95 °C and 1 min at 60 °C. Analysis used the sequence detection software supplied with the instrument. The relative quantification value is expressed as 2 ± DCT, where DCT is the difference between the mean CT value of triplicates of the sample and of the GAPDH control.

2.19. Western blots for transcription factors and Th2 cytokines

The expression levels of Th1/Th2 transcription factors (GATA-3, SOCS3, c-maf, STAT6, T-bet, Fox P3), cytokines (IL-4, IL-5, IL-13 and IL-10), Src kinases (Lyn and syk) and mast cell receptor (FcεRI) in the intestinal proteins of N-CPA and G-CPA treated mice were analyzed by immunoblotting using their specific antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) as described earlier (Kumar, Sharma, Singh et al., 2014). In brief, tissue proteins (50 µg/lane) were loaded and then resolved over a 12% SDS-PAGE gel electrophoresis and samples were then electrotransferred onto PVDF membranes (Immobilon-P, Millipore, Darmstadt, Germany) using a semi-dry blotting unit (Amersham Biosciences). Nonspecific binding sites on the blot (strip) was blocked with 3% BSA (in PBS-T) for 2 h at 37 °C. The blot was then washed in PBS-T, five times, before being coated with their respective above mentioned antibodies (Santa Cruz Biotechnology; each at 1:200 dilution) in PBS-T containing 3% BSA and placed at 37 °C for 2 h. Thereafter, the blot was washed with PBS-T five times and incubated for 2 h at 37 °C in a solution of HRP conjugated secondary antibodies (Sigma, 1:1000 dilution). Subsequently, after five washes with PBS-T, presence of antibodies on each blot was developed using an enhanced chemiluminescence system (Pierce, Waltham, MA). The β-actin was chosen as an endogenous control. Images of the bands were captured using a Syngene gel documentation system equipped with a CCD camera (Syngene) and densitometric analyses were then done using Gene tools software.

2.20. Statistical analysis

The data are expressed as mean ± 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. Purification of chickpea albumin and SDS PAGE analysis

The SDS-PAGE of CP-CPE showed the presence of a wide range (170–10 kDa) of proteins, including target albumin protein (26 kDa) (Fig. 1a). The purified albumin protein of approximately Mol. wt. 26 kDa appeared in the unbound fraction as a single band on 12% SDS-PAGE (Fig. 1) and purity was confirmed as a single peak at retention time 11.456 min on reverse phase-HPLC (Fig. 1b). Further, purified protein showed the IgE binding capability when incubated with pooled sera of sensitized mice, as well as chickpea allergic patient's sera (Fig. 1c, d). No IgE binding was detected with the healthy individual's sera as well as control mice sera. The chickpea purified protein was subjected to LC-MS/MS analysis and characterized as lectin protein, also reported as the crystal structure of a plant albumin from *Cicer arietinum* with Mol wt 26.1 kDa. The details of purified protein are given in [Supplementary Table S1](#). We further extended our experiment to assess the impact of glycation on the allergenicity of this purified protein using *in vivo* approach.

3.2. Altered secondary structure reflects functional role in suppression of allergic response

The CD profile of G-CPA showed alteration to its secondary structure compared to N-CPA group. Results of far-UV spectra clearly demonstrate that glycation reaction caused an increase in

the β-sheet conformation and decrease in alpha helix and random coil structures of chickpea albumin (Fig. 1e).

FTIR spectra of both N-CPA and G-CPA samples were recorded and analyzed for the peak frequency of amide I and amide II characteristics, indicating changes in structure of CP albumin attributed to secondary structural impairment. The FTIR spectra demonstrate the shifting of amide I peak to 1659 cm^{-1} in G-CPA from the native peak at 1650 cm^{-1} contributed by absorption of α-helix. Similarly amide II peak also shifted up to 1554 cm^{-1} in G-CPA sample in comparison to N-CPA peak 1548 cm^{-1} (Fig. 1f).

3.3. Estimation of free lysine residues

The free lysine residue in G-CPA sample was found to be $32.50 \pm 4.94\text{ nmol/mg}$ as compared to N-CPA $55.00 \pm 15.55\text{ nmol/mg}$ concentration.

3.4. Detection of Maillard reaction product

To detect the MR product formation in glycated protein sample, LC-MS analysis was carried out. Mass spectrometry result confirmed that under the examined conditions, the presence of glucose allows the formation of carboxymethyl lysine (CML) as indicated by a red solid arrow during glycol-conjugate preparation. The LC-MS spectra of glycated albumin samples along with standard (CML) were shown (Fig. 2a, b).

3.5. Allergic phenotype and body temperature after challenge

After the challenge, N-CPA treated mice showed visible illustrations of anaphylactic reactions, whereas suppression of systemic anaphylactic symptoms was found in G-CPA treated mice. Out of 5 mice from N-CPA group, 20% mice showed symptoms score 1, 40% mice showed symptoms 3, 20% mice showed symptoms 2, while 20% mice showed symptom 4. These mice showed a decrease of 4–5 °C in core body temperature. In G-CPA treated group, 20% mice showed symptom score 0, 60% mice showed symptom 1 and only 20% mice showed symptom 2. Most of G-albumin treated mice showed a nonsignificant drop of 1–2 °C in core body temperature (Fig. 2c & d).

3.6. Total IgE and chickpea albumin specific IgE and IgG1 levels

Significantly reduced level of total IgE and CP albumin specific IgE along with IgG1 were observed in the sera of G-CPA treated mice. The levels of specific IgE and IgG1 were observed to be significantly higher in N-CPA treated group as compared with the vehicle treated mice (Fig. 2e, f). The elevation of specific IgE and IgG1 was more on the 59th day of treatment with N-CPA group. However, glycated albumin sensitized group showed a significant decrease in specific IgE and IgG1 levels as compared to N-CPA group. Further, the level of total IgE was also reduced in the sera of G-CPA treated mice as compared to N-CPA treated group (Fig. S1d).

3.7. Measurement of MCP-1, MCPT-1 and TSLP level in serum

Increased levels of MCPT-1 and MCP-1 were found in N-CPA treated mice sera as compared with the control group, whereas in G-CPA group the levels of these allergic mediators were significantly lower than the native albumin treated group (Fig. S1a, S1c). Similarly, TSLP levels were also decreased in glycated albumin treated group when compared to the native treated group (Fig. S1f).

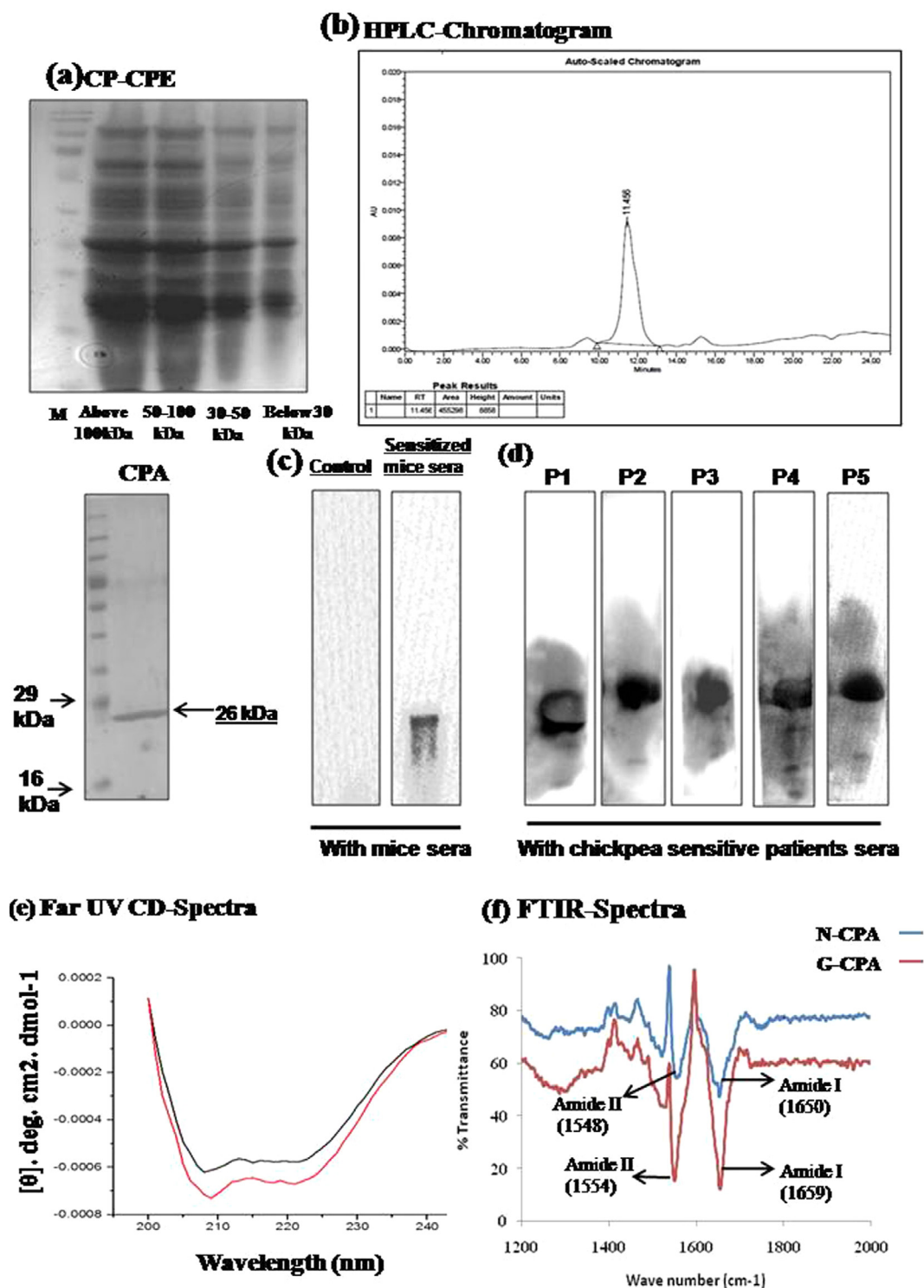


Fig. 1. Purification of IgE binding protein from chickpea seeds and IgE immunoblotting (a) SDS-PAGE showing protein profiles of chickpea crude protein extract (CP-CPE) and purified protein (b) Chromatogram of the 26 kDa purified protein by Reverse Phase High Pressure Liquid Chromatography (c & d) IgE immunoblotting of purified protein with sera of mice as well as chickpea allergic patients. (e) Far UV (black spectral line native, red spectral line glycated protein) and (f) FTIR spectra. Where M = Marker; kDa = kilo Dalton, CP-CPE = chickpea crude protein extract; CPA = chickpea albumin; IgE-I = IgE immunoblot; N-CPA = native chickpea albumin; G-CPA = glycated chickpea albumin.

3.8. The histamine and PGD2 levels in mice sera

The increased serum concentration of allergic mediators, namely histamine (17.5 ng/ml) and PGD2 (450 pg/ml), were found in the sera of N-CPA treated groups as compared to control. However, these mediators were showed a markedly decreased levels (Histamine-6.5 ng/ml, PGD2-130 pg/ml) in the sera of mice

sensitized with G-CPA as compared to N-CPA treatment group (Fig. S1b, S1e).

3.9. Cytokine production in supernatant of splenocyte culture

Purified CP albumin induced cytokine production from splenocyte was measured at 24 h, 48 h and 72 h time points. The elevated

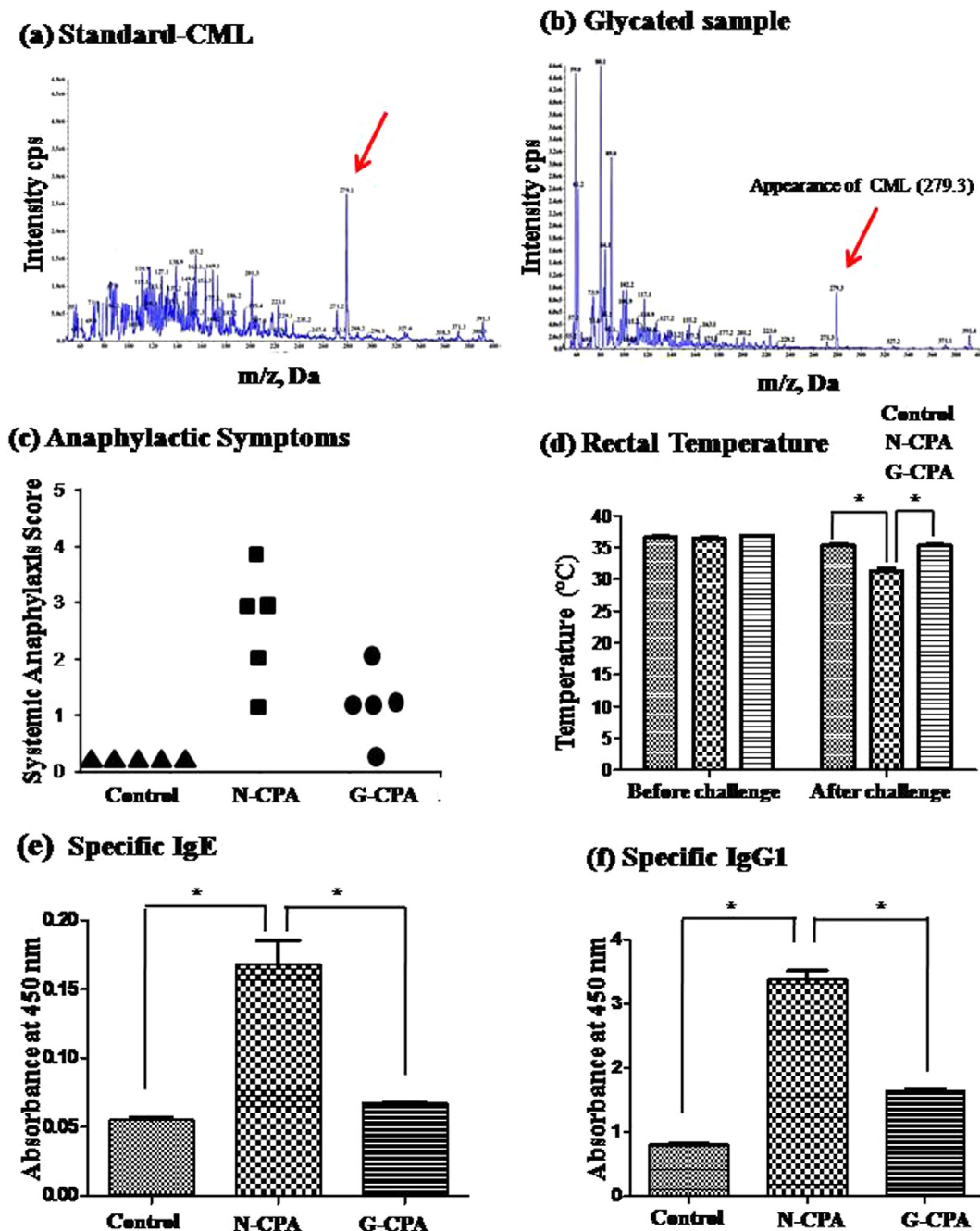


Fig. 2. (a) LC-MS spectral peak at 279.3, characteristics of standard CML and (b) identification of CML peak in glycated sample as indicated by red solid arrow. Allergic phenotype along with body temperature and specific IgE as well as IgG1 assay: (c) Anaphylactic symptoms scored in mice ($n = 5$ mice in each group) after 20–30 min following challenge with 5 mg N-CPA and (d) Rectal temperature was also measured on day 60 before and 20 min after challenge with 5 mg N-CPA; (e) Level of specific IgE in serum of control, N-CPA and G-CPA treated mice. (f) Specific serum IgG1 level of all the treatment groups. Data are presented in mean $\pm$ SEM; ($p < 0.05$) when compared to control with NCPA treatment and to N-CPA with G-CPA treatment. N-CPA = native chickpea albumin; G-CPA = glycated chickpea albumin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

production of IL-4, IL-17 and IL-10 were found in the splenocyte supernatant of N-CPA group as compared to control. The cytokine production was not in increasing order with their respective time points as cytokine IL-10 and IL-4 production were maximum in 72 h, whereas production of IL-17 was significantly increased at initial time points and remained elevated up to 72 h. In the splenocyte supernatant of G-CPA group, IL-2, IL-6 and IFN- γ production were significantly higher, while IL-4, IL-10 and IL-17 production were reduced in comparison to N-CPA group (Fig. 3a–f).

3.10. Histological expressions of Fox-P3, IL-4 and eosinophilic major basic protein

To determine the suppressing role of T-regulatory (T-reg) cells against the IL-4 mediated allergic response, the expression levels of both IL-4 and Fox-P3 in the lungs and intestine of both the native and glycated albumin treated groups were observed (Fig. 4). The result of Immunofluorescent histochemistry has shown a marked increase in the expression of Fox-P3 and reduced

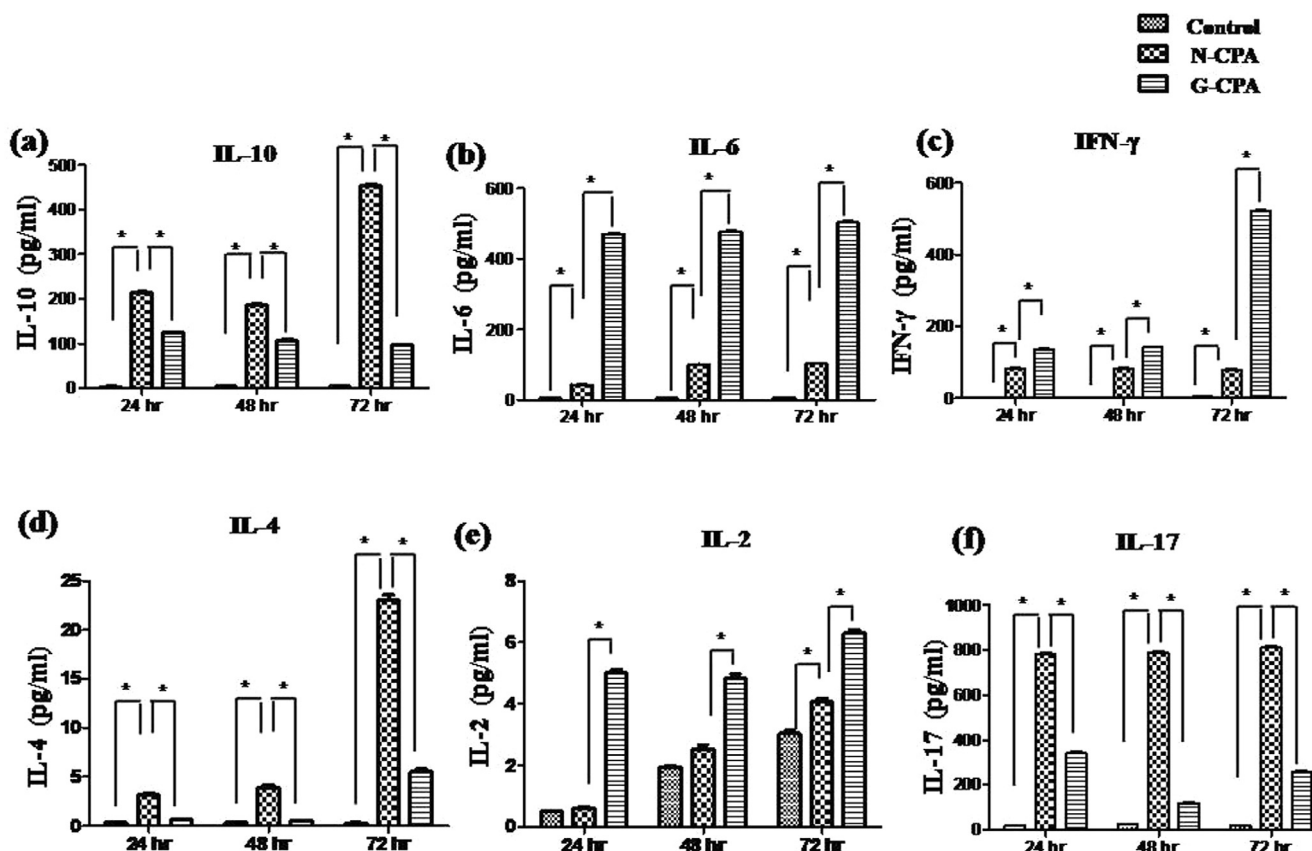


Fig. 3. Measurement of cytokines production ($n = 4$ mice in each group) in splenocyte culture supernatant at 24, 48 and 72 h after treatment of 100 μ g either N-CPA or G-CPA (a) IL-10 (b) IL-6 (c) IFN- γ (d) IL-4 (e) IL-2 and (f) IL-17. Data are presented in mean $\pm$ SEM, (* $p < 0.05$). N-CPA = native chickpea albumin; G-CPA = glycated chickpea albumin.

expression of IL-4 in glyco-conjugate of CP albumin treated group as compared to its native form. Further, as compared to native albumin, glycated CP albumin treated group showed a decrease in the tissue expressions of eosinophilic major basic protein (Fig. 5a).

3.11. Mast cell expansion in the lungs and intestine

The mast cell count was significantly higher in the lungs and intestine of purified albumin treated mice. However, expansion of mast cell was significantly lower in both the organs of G-CPA group than N-CPA (Fig. 5b).

3.12. Real-time PCR analysis of Th2 cytokines along with receptor and transcription factors

In the present study, the mRNA expressions of IL-4, IL4R (IL-4 receptor), GATA-3 and Foxp-3 were analyzed by Real time PCR technique in all the three groups. Significantly upregulated expressions of IL-4, IL-4R and GATA-3 were observed in the intestine of N-CPA in comparison to control, whereas no significant differences were observed in the mRNA levels of these cytokines and TF in G-CPA group when compared to control. However, mRNA expressions of above mentioned cytokines and TF were significantly down-regulated in G-CPA group when compared to the N-CPA treatment group. In the case of FoxP3, an up-regulated expression of Foxp3 was observed in the both N-CPA and G-CPA group over the control, while it was approximately 4 times down regulated in N-CPA group when compared with the G-CPA treatment (Fig. 6a–d).

3.13. Western blot analysis of intestinal protein

The immunoblot analysis of the intestinal proteins of all the treated groups was carried out. In the N-CPA treated group, an elevated protein expression of IL-4, IL-5 and IL-13 were observed as it was found 1.9, 2 and 1.6-fold higher, respectively in comparison to control group. Although, in the G-CPA group, these Th2 cytokines were significantly down regulated when compared to the N-CPA treated group. Similarly, expression levels of GATA-3 and SOCS-3 were 2 and 1.5-fold higher in N-CPA group in comparison to control, whereas both the TFs were observed with the reduced expressions in G-CPA treated group when compared with the native protein treated group. The expression levels of Fc ϵ RI, Lyn and Syk were 1.7, 1.5 and 1.7-fold higher in N-CPA treated group as compared to the control, while in the glycated albumin treated group, these molecules were down regulated in comparison to native group. In the case of T-bet, 0.5-fold decreased expression was found in N-CPA group, whereas the expression levels of FoxP3 and IL-10 were similar to the control group. However, protein expressions of FoxP3, T-bet and IL-10 were 1.5, 2 and 1.3-fold higher in G-CPA treated group when compared with the N-CPA treated group (Fig. 6e–l).

4. Discussion

Glycation process may be advantageous over other food processing methods as it alters the protein conformation, as well as the integrity of epitopes by glycation at specific amino acid residues. Glycation process induces a reversible or irreversible structural modification in the allergenic proteins, leading to protein

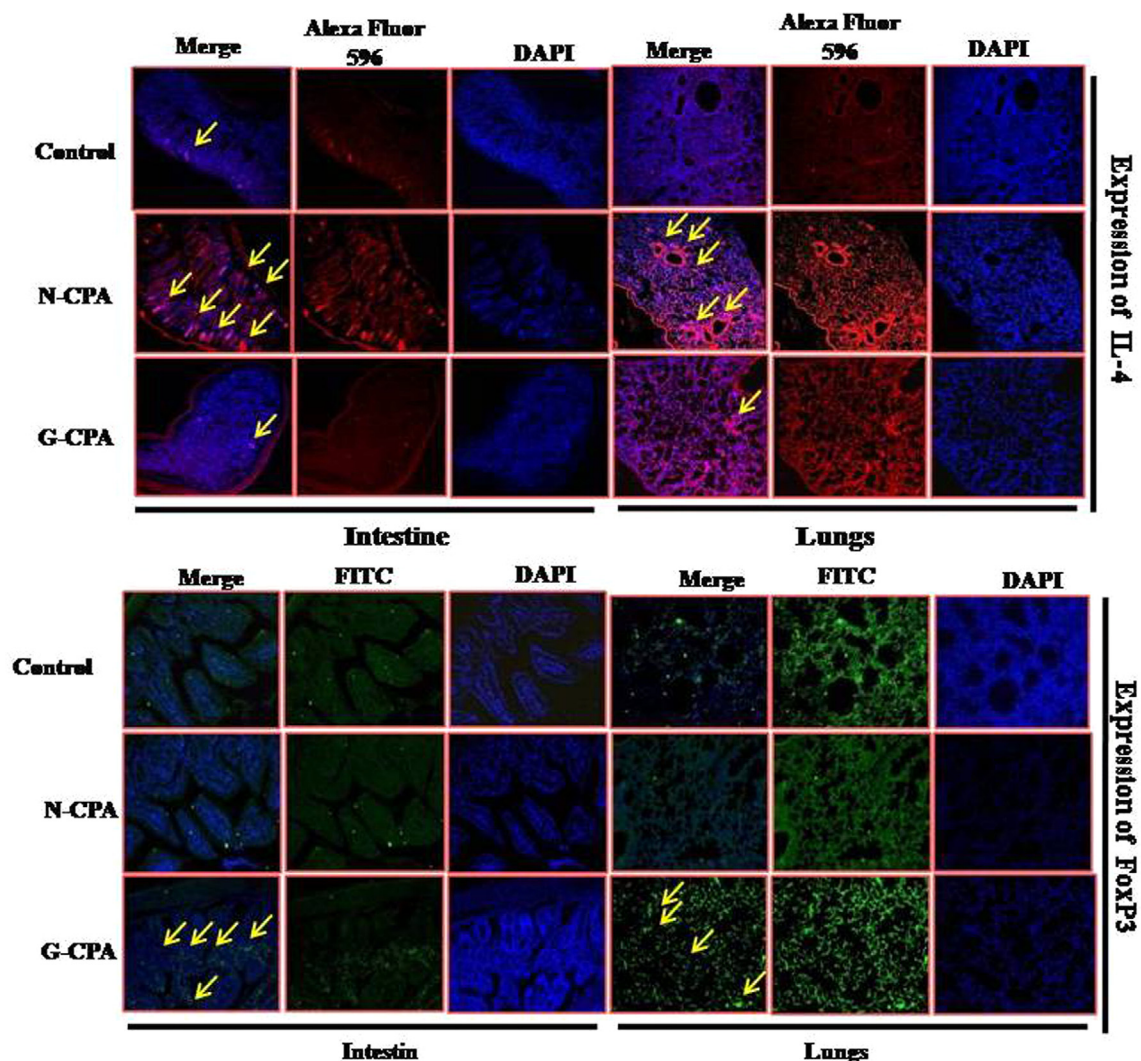


Fig. 4. Confocal images; The IL-4 and FoxP3 expression levels in the lungs and intestine of all treated groups (n = 4) as indicated by yellow solid arrow. N-CPA = native chickpea albumin; G-CPA = glycated chickpea albumin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

denaturation, aggregation; therefore, allergic behaviour of food protein may be attenuated, which ultimately reduces its allergenicity. In addition to this, glycation reaction may generate advance glycation end products (AGEs), which can abolish the antigen presentation by antigen presenting cells, and consequently suppression of allergic response (Gupta et al., 2016).

There are certain proteins having IgE binding domains suggested as allergens in a food. Among them seed storage proteins belonging to albumin family are considered as a chief source of food allergens, having the potential to elicit the IgE mediated allergic reactions (Sathe, Kshirsagar, & Roux, 2005). Many allergenic proteins from yellow (Sinapis alba, Sin a 1) and oriental mustard (Brassica juncea, Bra j 1), Brazil nut (Ber e 1), castor bean (Ricinus communis, Ric c 1) or white sesame seeds (Sesamum indicum, Ses i 1) have been classified as 2S albumins. A plant albumin from CP with mol. wt. of approximately 26 kDa on SDS-PAGE and 26.1 kDa by LC-MS/MS, was purified in this study. The clinical relevance of this allergen was demonstrated by IgE-immunoblotting with sera of chickpea nasobronchial allergic patients, indicating that CP 26 kDa protein may be responsible for allergic manifestations in sensitive individuals. In the present study, purified CP

albumin has shown similarity with lectin as sequence of this protein seems to be also very similar to lectin when the sequence was blast at NCBI. Apart from this, CP 26 kDa albumin was also reported with hemagglutination activity, corroborating its relation to lectin.

Like other foods, chickpea is also subjected to the various types of thermal processing prior to consumption. During thermal processing, generally a most frequent non enzymatic glycation reaction via MR takes place between food proteins/allergens and reducing sugars, resulting into glycation of participating food proteins/allergens. Recently, studies on the impact of glycation on several food allergens demonstrated that MR may either enhance or reduce allergenicity of food protein (Gupta et al., 2016). There are various studies demonstrating that the glycation of food protein can alter the allergenicity only in the terms of IgE binding property. Here, an attempt has been made to explore the detailed underlying mechanism behind the modulation in functionality of allergic protein in Balb/c mice.

The secondary structural changes of protein caused by glycation are effective ways of masking the allergenicity of the food allergens. Several studies have demonstrated that alteration in secondary structure of a protein may lead to suppression of the

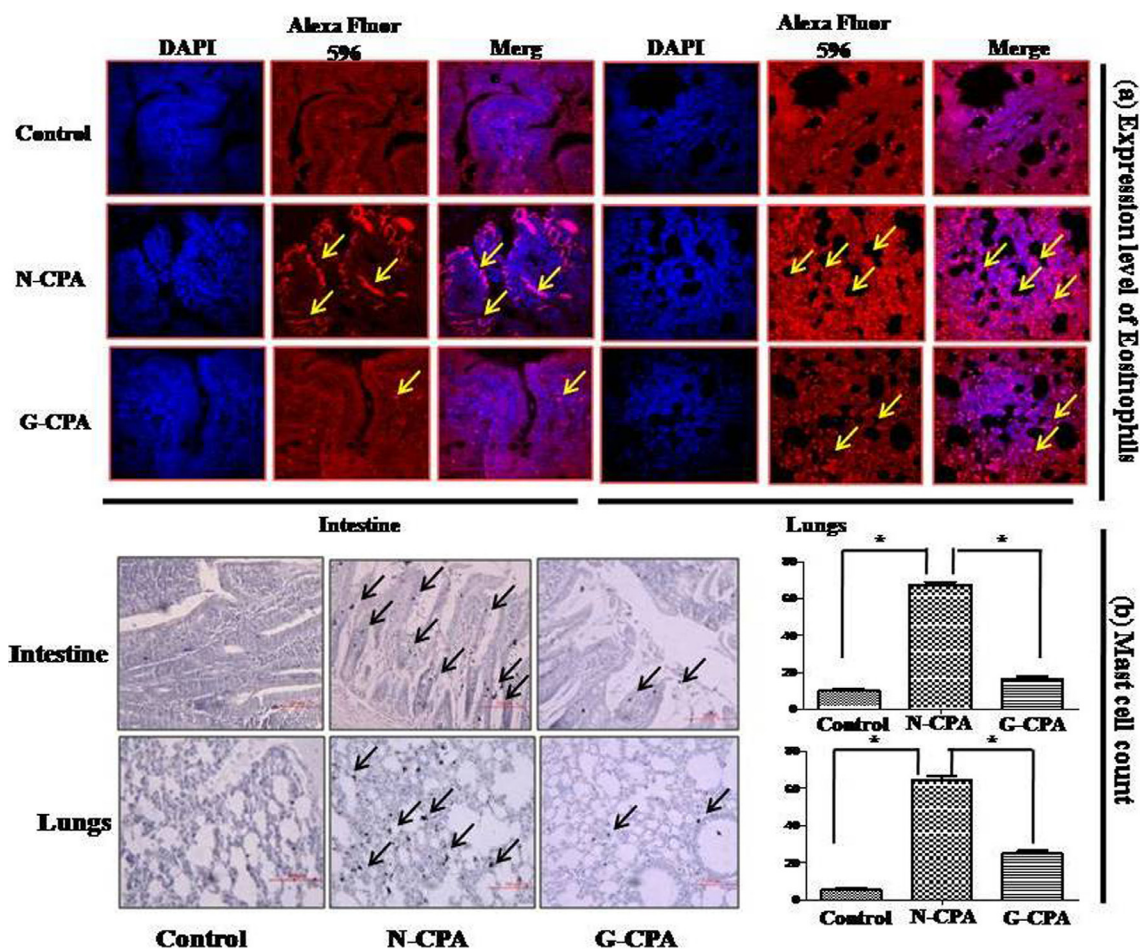


Fig. 5. Confocal images; (a) The eosinophil major basic protein (EMBP) expression in the lungs and intestine of all treated groups ($n = 4$ mice in each group) as indicated by yellow solid arrow. N-CPA = native chickpea albumin; G-CPA = glycated chickpea albumin. (b) Further, Toluidine blue staining for mast cell expansion in the lungs and intestine; The mast cell count was performed by three different individual in the given tissue area and presented as graphical forms. N-CPA = native chickpea; G-CPA = glycated chickpea albumin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

allergic response (Rupa, Nakamura, Katayama, & Min, 2014). In the present study, we observed the structure-function relationship of CP albumin upon glycation using Far UV CD spectroscopy and found that glycation caused the reduction in α -helix confirmation which may lead to a reduction in the allergenicity compared to native CP albumin. Further, we also analyzed the secondary structure using another technique Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy. FTIR spectra of both native and glycated CP albumin were recorded and analyzed for the peak frequency of amide I and amide II characteristics, which further proved the relationship between function and structure of CP albumin attributed to secondary structural impairment. In IR region, the band frequencies of amide I and II vibration are very sensitive to secondary structural impairment. The shifting of amide I arises (due to C=O stretching) and II peaks (due to N—H bending) in the present study depict the alteration in protein secondary structure upon glycation with D-glucose. The results of the present study demonstrate the shifting of amide I peak to 1659 cm^{-1} in glycated CP albumin from the native peak at 1650 cm^{-1} contributed by absorption of α -helix. Similarly, amide II peak also shifted to 1554 cm^{-1} in glycated albumin from native CP peak of 1548 cm^{-1} (Rigby, Marsh, Sancho, & Wellner, 2008). CD and FTIR results indicated towards the modification of purified protein with D-glucose. Further, we analyzed the final stage of MR and detected a well known MR product, namely CML in G-CPA sample by liquid chromatography mass spectroscopy. This finding sug-

gested that CP albumin modification arises due to the identified CML structure.

Furthermore, this study tested the hypothesis that whether the glycation influences the allergenicity induced by purified albumin (allergen) of CP. In the present study, purified albumin from CP was found to be capable to allergic sensitization as mice from N-CPA group showed visible anaphylactic sign and symptoms when challenged with the 5 mg concentration of purified protein on day 60. However, treatment with G-CPA was effective in suppressing the allergic clinical signs. The drop in body temperature is an established parameter to evaluate the anaphylactic reaction in the body (Sato et al., 2010). In our study, mice treated with native protein had a significant drop in body temperature [$4\text{--}5\text{ }^{\circ}\text{C}$] again validated the onset of systemic anaphylaxis after challenge. However, mice sensitized with G-CPA did not show a decrease in body temperature, suggesting that glycation of CP albumin reduced its sensitizing potential in Balb/c mice. Several reports based on food allergy have concluded that food allergens induce the cross linking of IgE bound to mast cells/basophils, and results in the release of various allergic mediators leading to allergic manifestation in a sensitized individual and mice (Kumar, Verma, Das, & Dwivedi, 2012). The serum levels of specific IgE antibodies were very low in mice that received i.p. immunization with a purified 26 kDa protein: i.e. only around OD 0.15 in ELISA. In contrast, the levels of specific IgG1 antibodies in the animals were high. It seems that anaphylactic reaction was induced by IgG1-mediated mechanism in the N-CPA

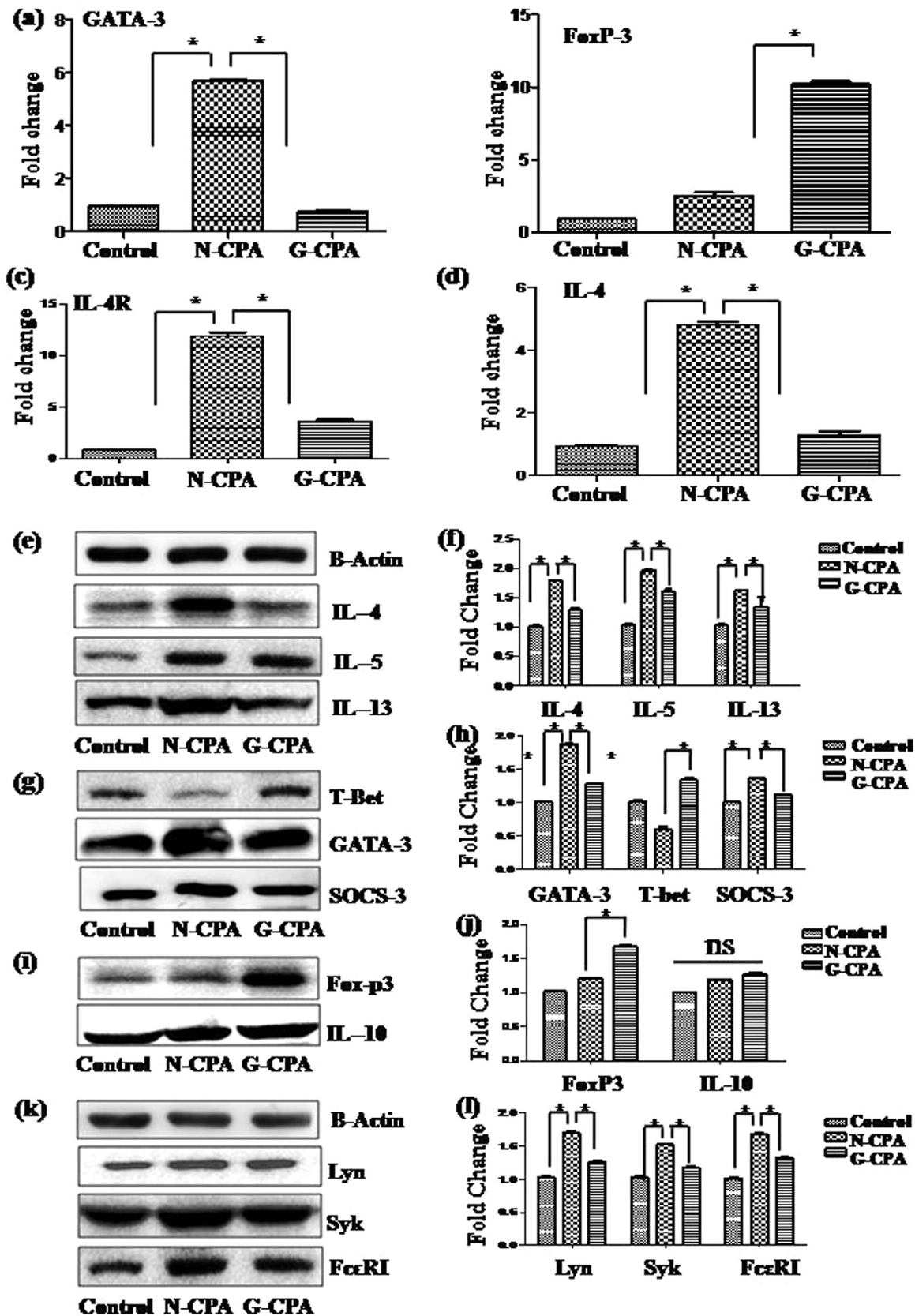


Fig. 6. Real time PCR results are showing mRNA expressions of (a) GATA-3 (b) FoxP3 (c), IL-4R (d) and IL-4 in both the treated group. Furthermore, western blots for the cytokines, Src kinases and transcription factors (e, g, i, k). Immunoblot results showing protein expressions of Th2 cytokines (IL-4, IL-5, IL-13, IL-10), associated transcription factor (GATA-3, T-bet and FoxP3) and Src kinases including mast cell receptor (Lyn, Syk, FcεRI) in different groups. Relative density (in fold terms) of each band measured by densitometry, showing in graph (f, h, j, l). The data from at least three repeated experiments are expressed as the mean $\pm$ SEM (Significant difference * $p < 0.05$).

treated group. Further, elevated serum levels of total IgE and various allergic mediators that include histamine, MCPT-1, MCP-1 and PGD2 were found in the N-CPA group as compared to control, whereas these allergic mediators and antibodies were substantially reduced in the sera of mice treated with G-CPA. These findings demonstrate that glycation of purified albumin suppresses its eliciting potency similar to other glycosylated food allergens towards the allergy by reducing the IgE production as well as germ line IgE class-switching and subsequently, inhibition of mast cell degranulation.

It is well known that cytokine IL-4 plays a crucial role in IgE mediated food allergy by inducing the class switching of IgE (Kumar et al., 2012). It was confirmed by an earlier study in which, IL-4 deficient Balb/c mice when sensitized and challenged with ovalbumin showed impaired IgE class switching due to IL-4 deficiency (Dourado et al., 2010). The cytokine IL-4 also promotes mast cell expansion in the intestine resulting into anaphylaxis in a murine model of food allergy (Burton et al., 2013). Thus, we analyzed the expression of IL-4 at histological level and found the enhanced protein expression of IL-4 as well as mast cell expansion in the lungs and intestine of mice sensitized with N-CPA, while IL-4 expression and mast cell distribution were significantly reduced in both the tissues of mice treated with the G-CPA. This result indicates that after glycation this protein may lead to suppression of allergy by downregulating both IL-4 expression and mast cell dispersion. Since induction of allergen-specific T-regs to develop the acquired tolerance is a critical event that leads to the development of a healthy immune response, therefore the contribution of T-regs to combat the allergenicity in mice treated with glycosylated albumin was also of interest in this study (Akdis & Akdis, 2009; Yamashita et al., 2004). T-reg cells express the transcription factor forkhead box P3 (FoxP3) and play an important role in the maintenance of intestinal homeostasis against allergic responses and production of antigen specific regulatory T-cells (Tsuji & Kosaka, 2008). Therefore, we observed the FoxP3 expression in mice in each treatment group. In this study, upregulated FoxP3 expression in the lungs and intestine of mice treated with glycosylated albumin was observed. This observation complements our other findings indicating that the increase in T-regs in this case may regulate the allergic response via the modulation of IL-4 cytokine. Further, the eosinophil levels in the lungs and intestine were investigated using EMBP expression to find out if there are any eosinophil mediated pathological conditions (Sharma et al., 2014). As expected, there was also an eosinophil mediated allergic reaction with N-CPA and G-CPA treatment was successful in overcoming the eosinophilic infiltration in the both targeted tissues. Mast cells are found throughout the body, but mainly dispersed on the epithelial surface of the respiratory tract, gastrointestinal tract, and genitourinary tract. Enormous infiltration of MCs may have an important role (by producing chemical mediators) in the progression of food allergic manifestations as found in various studies related to food allergy (Sharma et al., 2014). A massive infiltration of MCs was found in the lungs and intestine of mice treated with purified protein group indicating the severity of allergy. However, both the tissues from glycosylated protein treated group had less MCs expansion that also get support by reduced IL-4 cytokine expression in both the tissue as discussed before.

To analyze the purified albumin specific proliferation, we measured the cytokine production in splenocyte supernatant after 24, 48 and 72 h. Elevated concentrations of IL-6, IFN- γ and IL-2 as found in G-CPA treated group were able to regulate the production of IL-4, IL-17 and IL-10 cytokines than N-CPA group. These results clearly demonstrate the maintained homeostasis and balanced immune regulation caused by glycosylated CP albumin treatment.

The Th2 cytokines are major players in the induction of allergic response triggered by food allergens. Among them, cytokines IL-4,

IL-5 and IL-13 play an important role in allergic sensitization (Kumar et al., 2012; Wiedermann et al., 1988). Further, GATA-3 and SOCS-3 are two chief transcription factors (TFs) that are mainly involved in the development and maintenance of Th2 type immunity. Both the TFs promote the secretion of IL-4, IL-5 and IL-13 from Th2 cells (Kumar, Sharma, Singh et al., 2014; Yamashita, Takahashi, Tanaka, Nagai, & Inagaki, 2012). Thus, elevated protein expressions of IL-4, IL-5, IL-13 along with TFs GATA-3 and SOCS-3 in the intestinal protein of N-CPA group indicates towards the allergic potential of purified albumin via Th2 type immune response. However, up-regulated protein expression of T-bet and FoxP3 in G-CPA group demonstrate that glycation suppressed the allergic reaction (as reduced protein expressions of IL-4, IL-5 and IL-13 were observed) by induction of CP albumin specific T-reg cells and also by shifting the immune response towards Th1 from Th2 that balances the immune homeostasis. Among these cytokines and TFs, expression levels of IL-4 as well as its receptor (IL-4R), GATA-3 and FoxP3 were also analyzed at the mRNA level and found the same expression patterns as observed at protein level. The allergen specific IgE activates the MCs effectively via the high affinity receptor (Fc ϵ R1), which further activates several molecules of Src Kinase family, including Lyn and Syk (Gilfillan & Rivera, 2009). Thus, in the present study our interest was whether Src kinases are involved in the regulation of MCs degranulation. Reduced protein expressions of Fc ϵ R1 and Src kinase (Lyn) were found in G-CPA treated group as compared to N-CPA, suggesting the involvement of Lyn Src kinase in the down regulation of mast cell activation and Th-2 type immune responses.

5. Conclusion

In conclusion, this study provides the first detailed *in vivo* investigation that glycation of purified CP albumin with D-glucose attenuates the allergic potential of CP albumin. Suppression of allergic behaviour occurs by two important mechanisms that include oral tolerance induction by CP albumin specific T-reg cells and shifting of the immune response from Th2 to Th1 type. Overall, purified proteins having allergic potential may cause severe allergic reaction in sensitive individuals, separately and glycation of these allergenic proteins via MR could be an effective approach to combat the chickpea allergy.

Conflict of interest

Here, authors declared no conflict of interest.

Acknowledgment

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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.foodchem.2017.05.056>.

References

- Akdis, C. A., & Akdis, M. (2009). Mechanisms and treatment of allergic disease in the big picture of regulatory T cells. *Journal of Allergy & Clinical Immunology*, 123, 735–746.
- Ansari, N. A., Moinuddin, A. K., & Ali, A. (2009). Preferential recognition of Amadori-rich lysine residues by serum antibodies in diabetes mellitus: Role of protein glycation in the disease process. *Human Immunology*, 70, 417–424.
- Arif, B., Ashraf, J. M., Ahmad, J., Arif, Z., & Alam, K. (2012). Structural and immunological characterization of Amadori-rich human serum albumin: Role in diabetes mellitus. *Archives of Biochemistry and Biophysics*, 522(1), 17–25.
- Burton, O. T., Darling, A. R., Zhou, J. S., Noval-Rivas, M., Jones, T. G., Gurish, M. F., et al. (2013). Direct effects of IL-4 on mast cells drive their intestinal expansion and increase susceptibility to anaphylaxis in a murine model of food allergy. *Mucosal Immunology*, 6(4), 740–750.
- Davis, P. J., & Williams, S. C. (1998). Protein modification by thermal processing. *Allergy*, 53, 102–105.
- Dourado, L. P. A., da Silva Saldanha, J. C., Gargiulo, D. L., Noviello, M. D. L. M., Brant, C. C., Reis, M. L. C., et al. (2010). Role of IL-4 in aversion induced by food allergy in mice. *Cellular Immunology*, 262(1), 62–68.
- Gilfillan, A. M., & Rivera, J. (2009). The tyrosine kinase network regulating mast cell activation. *Immunology Review*, 228, 149–169.
- Gupta, R. K., Gupta, K., Sharma, A., Das, M., Ansari, I. A., & Dwivedi, P. D. (2016). Maillard reaction in food allergy: Pros and Cons. *Critical Reviews in Food Science and Nutrition*. <http://dx.doi.org/10.1080/10408398.2016.1152949>.
- Gupta, R. K., Gupta, K., Sharma, A., Das, M., Ansari, I. A., & Dwivedi, P. D. (2017). Look Insight: Health Risks and Benefits of Chickpea (*Cicer arietinum*) Consumption. *Journal of Agricultural and Food Chemistry*, 65, 6–22.
- Hauser, M., Egger, M., Wallner, M., Wopfner, N., Schmidt, G., & Ferreira, F. (2008). Molecular properties of plant food allergens: A current classification into protein families. *The Open Immunology Journal*, 1, 1–12.
- Houska, M., Setinova, I., Kucera, P. (2011). Food allergens and processing – review of recent results. 11th Intl. Congress on Engineering and Food; Athens, Greece, 22–26 May.
- Kumar, S., Sharma, A., Das, M., Jain, S. K., & Dwivedi, P. D. (2014). Leucoagglutinating phytohemagglutinin: Purification, characterization, proteolytic digestion and assessment for allergenicity potential in BALB/c mice. *Immunopharmacology & Immunotoxicology*, 36(2), 138–144.
- Kumar, S., Sharma, A., Singh, G., Verma, A. K., Roy, R., Gupta, R. K., et al. (2014). Allergenic 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 Research International*, 57, 130–141.
- Kumar, S., Sharma, A., Verma, A. K., Chaudhari, B. P., Das, M., Jain, S., et al. (2013). Allergenicity potential of red kidney bean (*Phaseolus vulgaris* L.) proteins in orally treated BALB/c mice and passively sensitized RBL-2H3 cells. *Cellular Immunology*, 284(1), 37–44.
- Kumar, S., Verma, A. K., Das, M., & Dwivedi, P. D. (2012). Molecular mechanisms of IgE mediated food allergy. *International Journal of Immunopharmacology*, 13, 432–439.
- 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. *Journal of Allergy & Clinical Immunology*, 106(1), 150–158.
- 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. *Clinical & Experimental Allergy*, 41(8), 1157–1168.
- Mondoulet, L., Paty, E. B., Drumare, M. F., Ah-Leung, S., Scheinmann, P., Willemot Wal, R. M., et al. (2005). Influence of thermal processing on the allergenicity of peanut proteins. *Journal of Agricultural and Food Chemistry*, 53(11), 4547–4553.
- Pastorello, E. (2004). Allergy to peanuts. *EFSA Journal*, 34, 76–84.
- Rachwat, D., Nebesny, E., & Budryn, G. (2013). Chickpeas – composition, nutritional value, health benefits, application to bread and snacks: A review. *Critical Reviews in Food Science and Nutrition*. <http://dx.doi.org/10.1080/10408398.2012.687418>.
- Rigby, N. M., Marsh, J., Sancho, A. L., & Wellner, K. (2008). The purification and characterisation of allergenic hazelnut seed proteins. *Molecular Nutrition in Food Research*, 52(2), S251–S261.
- Rupa, P., Nakamura, S., Katayama, S., & Min, Y. (2014). Effects of ovalbumin glycoconjugates on alleviation of orally induced egg allergy in mice via dendritic-cell maturation and T-cell activation. *Molecular Nutrition in Food Research*, 58, 405–417.
- Sathe, S. K., Kshirsagar, H. H., & Roux, K. H. (2005). Advances in seed protein research: A perspective on seed allergens. *International Journal of Food Science & Technology*, 70(6), r93–r120.
- Sampson, H. A. (2004). Update on food allergy. *Journal of Allergy & Clinical Immunology*, 113, 805–819.
- Sato, Y., Akiyama, H., Matsuoka, H., Sakata, K., Nakamura Ishikawa, R., et al. (2010). Dietary carotenoids inhibit oral sensitization and the development of food allergy. *Journal of Agriculture & Food Chemistry*, 58, 7180–7186.
- Sharma, A., Kumar, S., Gupta, R. K., Roy, R., Gupta, K., Verma, A. K., et al. (2014). Elucidation of immediate type I reactions in native and GM mustard (*Brassica* spp.). *Food Research International*, 64, 810–821.
- Shriver, S. K., & Yang, W. W. (2011). Thermal and nonthermal methods for food allergen control. *Food Engineering Revision*, 3, 26–43.
- Tsuji, N. M., & Kosaka, A. (2008). Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. *Trends in Immunology*, 29, 532–540.
- Usui, M., Saito, A., Taniguchi, N., Nishijima, N., Azakami, H., & Kato, A. (2003). Reduction of antigenicity of Cry j 1, major allergen of Japanese cedar pollen, by the attachment of polysaccharides. *Journal of BioScience & Biotechnology*, 67(11), 2425–2430.
- 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. *Toxicology Letter*, 210(1), 24–33.
- Wal, J. M. (2003). Thermal processing and allergenicity of foods. *Allergy*, 58, 727–729.
- Wiedermann, U., Jahn-Schmid, B., Fritsch, R., Bauer, L., Renz, H., Kraft, D., et al. (1988). Effects of adjuvants on the immune response to allergens in a murine model of allergen inhalation: Cholera toxin induces a Th1-like response to Bet v 1, the major birch pollen allergen. *Clinical & Experimental Immunology*, 111, 144–151.
- Yaldagard, M., Mortazavi, S. A., & Tabatabaie, F. (2008). The principles of ultra high pressure technology and its application in food processing/preservation: A review of microbiological and quality aspects. *African Journal of Biotechnology*, 7, 2739–2767.
- Yamashita, H., Takahashi, K., Tanaka, H., Nagai, H., & Inagaki, N. (2012). Overcoming food allergy through acquired tolerance conferred by transfer of Tregs in a murine model. *Allergy*, 67, 201–209.
- Yamashita, M., Ukai-Tadenuma, M., Miyamoto, T., Sugaya, K., Hosokawa, H., Hasegawa, A., et al. (2004). Essential role of GATA3 for the maintenance of type 2 helper T (Th2) cytokine production and chromatin remodeling at the Th2 cytokine gene loci. *Journal of Biological Chemistry*, 279(26), 26983–26990.

Figure S1

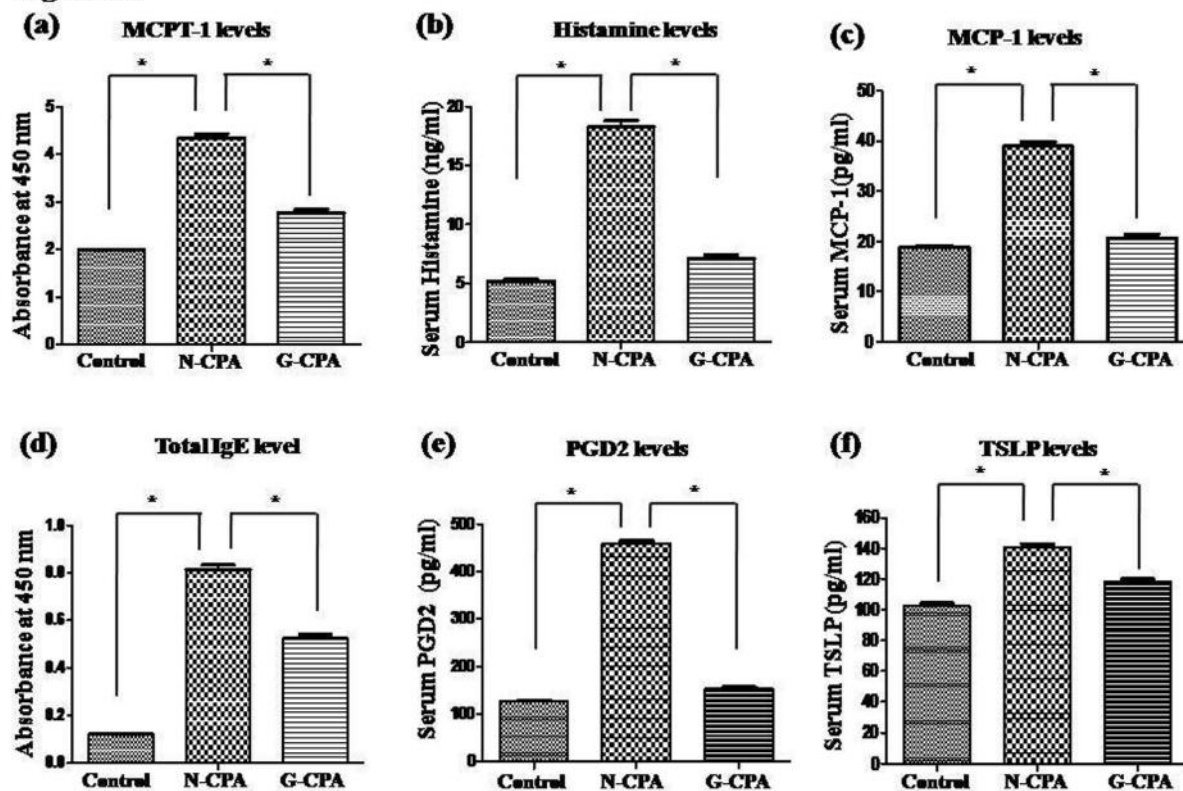


Figure S1: Serum levels of various allergic mediators in pooled sera of each group mice (a) MCPT-1 (b) Histamine (c) mMCP-1 (d) Toatal IgE (e) PGD2 and (f) TSLP. Data are presented in mean $\pm$ SEM, (* $p < 0.05$). N-CPA= native chickpea albumin; GCPA=glycated chickpea albumin.

11 Table S1: LC-MS/MS analysis of purified protein from chickpea

Protein sequence	Sequence similarity with proteins and their accession	Score	Mol. wt. (kDa)
MAKTGYINAAFRSSQNNE AYLFINDKYVLLDYAPGT SNDKVLYGPTPVRDGFKS LNQTVFGSYGVDCSFDTD NDEAFIFYEKFCALIDYA PHSNKDKIILGPKKIADM FPFFEGTVFENGIDAAYR STRGKEVYLFKGDQYARI DYETNSMVNKEIKSIRNG FPCFRNTIFESGTDAAFA SHKTNEVYFFKGDYYARV TVTPGATDDQIMDGVRKT LDYWPSLRGIIPLEN	Lectin OS Cicer arietinum GN CAL PE 2 SV 1; R4NHA7	231	26.1

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