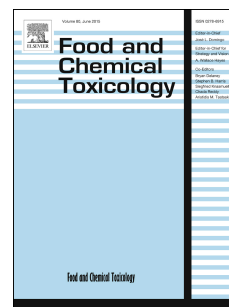


Accepted Manuscript

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PII: S0278-6915(15)30040-5

DOI: [10.1016/j.fct.2015.08.018](https://doi.org/10.1016/j.fct.2015.08.018)

Reference: FCT 8375

To appear in: *Food and Chemical Toxicology*

Received Date: 5 May 2015

Revised Date: 12 August 2015

Accepted Date: 19 August 2015

Please cite this article as: Mishra, A., Gaur, S.N., Lavasa, S., Arora, N., In vitro assessment of allergenicity features and localization of probable IgE binding regions, *Food and Chemical Toxicology* (2015), doi: 10.1016/j.fct.2015.08.018.

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***In vitro* assessment of allergenicity features and localization of probable IgE binding regions**

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ABSTRACT

Rice is cultivated as a staple grain crop in many countries, especially in Asia. In the present study, recombinant rice chitinase was expressed, purified and characterized by *in silico* and immunobiochemical methods. Rice chitinase was affinity purified and it resolved at 24 kDa on SDS-PAGE. Purified protein was analyzed for pepsin resistance, heat stability, and IgE binding using atopic patients' sera. Chitinase was resistant to pepsin digestion and heat treatment at 90°C for 1 h. It showed significant IgE binding with 7 of 110 patients' sera positive to different food allergens. Homology modeled 3D structure of rice chitinase was used for B cell epitope prediction. *In silico* predicted B cell peptides were assessed for IgE binding by ELISA using food allergic patients' sera, epitope RC2 showed IgE binding comparable to chitinase. In conclusion, chitinase was identified as a potential allergen and may share cross reactive epitopes with food allergens.

Keywords: B-cell epitopes, food allergen, rice (*Oryza sativa*), chitinase, homology modeling, *in silico* tools

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Rice is cultivated as a staple grain crop in many countries, especially in Asia. In the present study, recombinant rice chitinase was expressed, purified and characterized by *in silico* and immunobiochemical methods. Rice chitinase was affinity purified and it resolved at 24 kDa on SDS-PAGE. Purified protein was analyzed for pepsin resistance, heat stability, and IgE binding using atopic patients' sera. Chitinase was resistant to pepsin digestion and heat treatment at 90°C for 1 h. It showed significant IgE binding with 7 of 110 patients' sera positive to different food allergens. Homology modeled 3D structure of rice chitinase was used for B cell epitope prediction. *In silico* predicted B cell peptides were assessed for IgE binding by ELISA using food allergic patients' sera, epitope RC2 showed IgE binding comparable to chitinase. In conclusion, chitinase was identified as a potential allergen and may share cross reactive epitopes with food allergens.

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Highlights:-

- Expression and purification of rice chitinase protein for immunobiochemical studies
- 3D structure of rice chitinase was generated by homology modeling
- *In silico* identification of B-cell epitopes of rice chitinase
- Predicted B-cell epitopes were validated by *in-vitro* IgE binding studies
- Rice chitinase identified as a potential allergen by *in silico* and *in vitro* studies

1. Introduction

Rice is cultivated as a staple grain crop in many countries, especially in Asia. Although, food allergy to rice is rare, the prevalence of IgE-mediated rice allergy is about 0.8% in subjects with asthma and rhinitis in India (Kumar et al., 2007) and 10% in atopic subjects in Japan (Ikezawa et al., 1992). Rice allergy is more prominent in adults than in children. Chitinases belong to the family of pathogenesis-related (PR) proteins and provides defense against fungal infection to the host plant. Rice chitinase has been isolated and expressed as transgenic protein in many food crops but the allergenic potential needs to be evaluated. Previous study of our laboratory has shown that rice chitinase shares significant homology with allergens from *Hevea brasiliensis*, *Castanea sativa*, *Persea americana* etc. by *in silico* methods (Mishra, Gaur, Singh, & Arora, 2012). Proteins sharing similar primary structure tend to share three dimensional structural motifs and are more likely to share allergenic cross reactive epitopes. However, more information needs to be gathered from the epitopic regions of chitinase protein, in studying the clinically important cross reactivity and for engineering hypoallergenic variants with reduced IgE binding (Schein, Ivanciuc, & Braun, 2007).

Epitope mapping elucidates the antigenic determinants involved in immunoglobulin binding reactions (Nair, Kukreja, Singh, & Arora, 2011). These antigenic determinants are the factors responsible for eliciting different types and intensities of the immune responses in an allergic cascade. Among different strategies employed for epitope mapping, *in silico* methods possess an edge over others as they are less time consuming and quite reliable (Sharma, Gaur, & Arora, 2013). In case of food allergens both linear and conformational IgE binding epitopes, are of importance as linear epitopes undergo digestion, while conformational epitopes are involved in oral allergy syndrome (Pomes A, 2010). The present study aimed at expressing, purifying and

characterizing rice chitinase by immunobiochemical methods. Further, homology modeled 3D structure of rice chitinase was used for B cell epitope prediction and synthetic peptides representing the predicted epitopes were tested for IgE binding by ELISA using food allergic patients' sera.

2. Materials & Methods

2.1 Sequence alignment for Chitinase: The sequence homology of chitinase (accession number: AAC37516) was analyzed using two allergen databases i.e. Structural Database of Allergenic Proteins (<http://fermi.utmb.edu/SDAP/>) and Food Allergy Resources and Research Program (Food Allergy Research and Resource Program (FARRP) AllergenOnline database version 15.0 updated on 12 January 2015). Sequence alignment of >35% identity, through a window of 80 amino acids, with known allergen(s), was considered significant as per the proposed guidelines (FAO/WHO, 2001 and Codex, 2003).

2.2 Chitinase gene: For subcloning of chitinase gene in an expression vector of interest (pET28a+), restriction analysis was done and Hind III & Bam HI restriction enzymes were selected for cloning. Five nanograms of recombinant plasmid containing the chitinase gene was obtained from BIOGENE Co. and digested with Hind III & Bam HI. The digested product was run on the agarose gel and eluted using QIAquick gel extraction kit (Qiagen, Germany). Further, vector backbone was prepared by digesting pET28a+ expression vector. The digested insert and vector were ligated at 16°C for 16 h, and transformed into DH5α *Escherichia coli* cell. Individual colonies were picked and grown overnight at 37°C to isolate plasmid. It was digested with restriction enzymes, and a positive clone containing the gene was sequenced.

2.3 Expression and purification of recombinant *Oryza sativa* chitinase: The plasmid containing the insert was transformed into BL-21 *E. coli* cells. A clone containing the plasmid was grown until 0.6 - 0.8 OD at 600nm induced with 1mM isopropyl- β -D-thiogalactopyranoside for 3 h and harvested. The pellet was suspended in binding buffer containing 50 mM NaH_2PO_4 , 300 mM NaCl, and 10 mM imidazole (pH 8.0), sonicated, and centrifuged. The supernatant was incubated for 2 h with 2 mL of Ni-NTA (nickel-nitrilotriacetic acid) equilibrated with binding buffer. The column was washed with buffer containing 20 mM imidazole, 300 mM NaCl, and 50 mM NaH_2PO_4 , and bound proteins were eluted using buffer containing 250 mM imidazole, 300 mM NaCl, and 50mM NaH_2PO_4 . The purified protein was resolved on 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), transferred to nitrocellulose membrane, and probed with antibodies raised in mice against chitinase as described earlier (Singh, Singh, Prasad, Gaur, & Arora, 2008).

2.4 Immunoblot: Recombinant rice chitinase was resolved on a 12% SDS-PAGE **under reducing conditions** and protein band was electrophoretically transferred onto a Nitrocellulose (NC) membrane (Sigma, USA). The un-reacted sites on the NC membrane were blocked with 3% defatted milk in PBS, and **incubated with pooled patients' sera positive to different food allergens (1:10 v/v), antibodies raised in mice against chitinase (1:10 v/v) and His-tag antibody (1:5000 v/v) obtained from SIGMA–Aldrich (St. Louis, MO, USA), respectively**, at 4°C for overnight. After washing with phosphate-buffered saline (PBS) further incubated with **anti-human IgE (HRP conjugated), anti-mouse IgG-horse radish peroxidase (HRP) (1:1000 v/v) and PBS, respectively, and developed using 3, 3'-Diaminobenzidine (Sigma, USA).**

2.5 Heat stability studies: The effect of temperature on stability of purified chitinase protein was determined by a previously described protocol (Herouet et al., 2005). **Briefly, chitinase protein was dissolved in 20mM Tris-HCL and 5 mM EDTA buffer at a concentration of 0.025 mg/mL and heated at 90°C for 15, 30, 45, and 60 min in a temperature-controlled heating block. The assay was terminated by ice cooling of samples, and the reaction was stopped by adding sample buffer. The heat treated protein was analyzed by SDS-PAGE and Western blot for comparison with untreated sample.**

2.6 Digestibility studies: The digestibility of chitinase protein was determined by incubation in simulated gastric fluid (SGF), that is, 3.2 mg/mL pepsin in 0.03 M NaCl at pH 1.2. Chitinase protein (400 µg) was dissolved in 400 µL of SGF. Digestion was carried out at 37°C, and 30 µL of the samples were aliquoted at 0, 5, 15, 30, 45, and 60 mins. The reaction was stopped by adding 26 µL of sample buffer. The mixture was then boiled for 5 min and was analyzed by SDS-PAGE.

2.7 Allergen Extracts: Food extracts for allergy diagnosis were procured from All Cure Pharma Private Limited, New Delhi, approved by Drug Controller.

2.8 Sera Collection: Patients with allergic rhinitis and asthma aged 18-50 years were skin prick tested (**SPT**) with various fungi, pollens, insects, and food allergen extracts to detect sensitization. The diagnosis of asthma and rhinitis was ascertained following American Thoracic Society Guidelines (1991) (American Thoracic Society Lung Function Testing: selection of reference values and interpretative strategies, 1991) and Allergic Rhinitis and its Impact on

Asthma guidelines (Bousquet, Cauwenberge, & Khaltaev 2001), respectively. Patients tested positive by skin prick and having any two of the symptoms viz. anaphylaxis, redness of mouth, urticaria, nausea, vomiting, diarrhea, abdominal cramps, running nose or breathlessness, after ingestion of food extracts were recruited in the study. Blood samples were collected from **patients showing marked positive skin reactions to different food extracts at the US Lavasa Medical and Research Centre, Chandigarh, and the outpatient department, V.P. Chest Institute, Delhi, a referral chest hospital. All the patients' were SPT positive to one or the other food allergen and specifically – patient no. 1 & 4 were SPT positive to corn, patient no. 2 & 6 were SPT positive to mustard, patient no. 3 & 5 were SPT positive to rice, respectively, while patient no. 7 was SPT +ve to both tomato and mustard. The sera samples have been assessed by ELISA and were positive to the corresponding food allergen as mentioned in Table 1.** The study protocol was approved by Human Ethics Committee of CSIR - Institute of Genomics and Integrative Biology, Delhi.

2.9 Estimation of specific IgE: Levels of specific IgE in skin prick test positive patients' sera was determined by enzyme linked immunosorbent assay (ELISA) following the protocol of Singh et al. (Singh et al., 2006). Briefly, 250 ng of chitinase was coated in carbonate buffer (250ng/100µl per well) overnight at 4°C in a microtitre plate (Nunc, USA). After washing with PBS–Tween 20 (0.05%), the nonspecific sites were blocked with 3% defatted milk for 3 h at 37°C. The plate was washed again and incubated with 1:10 v/v diluted sera of SPT positive patients, overnight at 4°C. Normal human sera (n = 3) served as control. The plate was washed and incubated with antihuman IgE-horse radish peroxidase (1:1000 v/v; Sigma Chemical Co., St

Louis, MO, USA) for 3 h at 37°C. Color was developed with orthophenylene diamine. The reaction was stopped by adding 5 N H₂SO₄ and the absorbance was read at 492 nm.

2.10 ELISA inhibition: ELISA inhibition was performed to determine the allergenic potency of the recombinant rice chitinase. Briefly, microtitre plate was coated with purified recombinant chitinase protein (100 ng) and blocked with 3% defatted milk. The plate was incubated with pre-incubated mixture (1:10 v/v) of chitinase +ve pooled patients' sera (n=7) and graded amounts of chitinase 0.1, 1, 10, 100, and 1000ng as inhibitor. Sera of non-allergic subjects were used as control. The remaining steps were the same as followed for ELISA. Inhibition was presented as percentage decrease in absorbance of sample with inhibitor to that of sample without inhibitor and allergenic potency was defined by establishing the concentration of protein required for 50% inhibition.

2.11 Prediction of IgE binding epitopes of chitinase

2.11.1 Sequence retrieval - The amino acid sequence for rice chitinase accession no. (AAC37516) was retrieved from NCBI (www.ncbi.nlm.nih.gov) (Kim et al., 1998).

2.11.2 Prediction of linear epitopes – ABCpred (Saha & Raghava, 2006), BCPred (El-Manzalawy, Dobbs, & Honavar, 2008a; El-Manzalawy, Dobbs, & Honavar, 2008b), BepiPred 1.0b (Larsen, Lund & Nielsen, 2006), BcePred (Saha, & Raghava, 2004) and DNASTAR Lasergene version 7.2 (DNASTAR Inc., Madison, WI, USA) web servers were employed to predict linear B-cell epitopes. The web servers used primary sequence of the query protein as input for prediction studies and these web-servers are based on the concepts of artificial neural networks, support vector machine, hydrophilicity scale with a hidden markov

model and amino acid propensity scales of Hopp -Woods (hydrophilicity), Emini (Surface probability), Jameson-Wolf (antigenic index) and Karplus-Schulz (flexibility). For selection of consensus epitopes, a preference was given to residues predicted by tools having an area under receiver operating characteristic curve (AUC) greater than 0.70. Regions recognized by three or more tools with default settings were selected.

2.11.3 Homology modeling and prediction of conformational epitopes - *O. sativa* chitinase sequence was searched for homology in the Protein Data Bank (PDB) and homologous template was selected by BLAST server (<http://ncbi.nlm.nih.gov/>). ClustalW multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/clustalw/>) with default settings was used to align the query and the template protein. Alignment data and crystal structure of the template were submitted to academic version of Modeller 9.11v for generation of 3D model. 400 models were generated for chitinase and evaluated using Discrete Optimized Protein Energy (DOPE) method assessment functions of MODELLER. Minimum DOPE score model was selected for stereo-chemical analysis. The incorrectly modeled loop regions were identified and refined by loop.py command, 50 more models were generated after loop refinement. And the final model was selected for energy minimization and quality assessment having minimum DOPE score.

The refined model was optimized by steepest descent algorithm of the Amber force field implemented in DeepView/Swiss-PDBViewer v4.1.0. Stereo-chemical quality and reliability of the model was assessed by several structure assessment methods like PROCHECK, (http://nihserver.mbi.ucla.edu/SAVES_3/) and ERRAT (<http://nihserver.mbi.ucla.edu/>). The refined structure for *O. sativa* chitinase was visualized using Jmol (<http://www.jmol.org/>) software. 3D structure was submitted to Disco Tope 2.0

Server, and CBTOPE web servers for prediction of conformational epitopes. For selection of consensus epitopes, a preference was given to residues predicted by tools having an area under receiver operating characteristic curve (AUC) greater than 0.70. Regions recognized by two or more tools with default settings were selected for the study.

2.12 Peptide synthesis: Three predicted epitopes (peptides) were synthesized commercially (ThermoFisher SCIENTIFIC, Germany) with a purity of >95% and their sequences were confirmed by mass spectrometry (MALDITOF) analyses (Supplementary Table 1).

2.13 IgE binding of the peptides: Specific IgE against the synthetic peptides was determined and compared with chitinase protein by enzyme-linked immunosorbent assay (ELISA). The microtiter plate (Nunc, USA) was coated with 250 ng/100 μ l/well of recombinant chitinase or 100 ng/100 μ l/well of synthetic peptides in carbonate-bicarbonate buffer (pH 9.6). After, washing with phosphate-buffered saline PBS-Tween 20 (0.05%), the non-specific sites were blocked with 3% defatted milk for 3 h at 37°C. After washing the plate with PBST, individual patients' sera (1:10 v/v) from food allergic patients' positive to chitinase were added and the plate was incubated overnight at 4°C. The IgE binding was probed with anti-human IgE horseradish peroxidase (1:1000 v/v; Sigma, USA). The color was developed with orthophenylenediamine and hydrogen peroxide in citrate buffer. The absorbance was read at 492 nm after stopping the reaction with 5 N sulphuric acid. Sera from 7 healthy subjects were used as control.

2.14 Peptide similarity search of the predicted linear epitopes: Linear epitopes predicted for rice chitinase were assessed for similarity with the peptides of known allergens in SDAP database (Ivanciuc et al., 2009). PD values less than 7.0 were included in the study.

2.15 Statistical analyses: Statistical analysis of the results was done using GraphPad Prism and GraphPad InStat software (GrapPad Software, San Diego, CA, USA). For peptide ELISA the significance was calculated using one-way analysis of variance (ANOVA) followed by Dunnett's comparison test between control and experimental groups. A 'p' value ≤ 0.001 was considered as statistically significant.

3 RESULTS

3.1 Homology of Chitinase with different allergens: Allergen homologues of chitinase protein were identified using allergen databases. In full length FASTA alignment, in Structural Database of Allergenic Proteins (SDAP) database, chitinase showed more than 60% identity with three reported allergens (Figure S3; Supplementary Table 4). And, the same was observed for Farrp allergen database, chitinase showed maximum identity (63.80%) with *Hevea brasiliensis* (class I chitinase) followed by *Castanea sativa* (chitinase Ib) 63.40% and *Persea americana* (endochitinase) 63.10% (Supplementary Table 4). The 80 amino acid sliding window search showed that chitinase sequence shares high identity (>35%) with chitinase from *H. brasiliensis*, *Persea americana* (avocado) and *Castanea sativa* (chest nut) allergens (Supplementary Table 4).

3.2 Expression and purification of rice chitinase: Recombinant rice chitinase was expressed in *E. coli* BL21 (DE3) cells and affinity purified to homogeneity. The yield of the purified chitinase protein was approximately 1.50mg/L of bacterial culture and the recombinant protein resolved at 24 kDa on a 12% SDS-PAGE. **The recombinant protein was recognized by pooled patients' sera, mice raised antibodies against chitinase and His-tag antibody, respectively, on immunoblotting (Figure 1).**

3.3 Thermal stability: The untreated chitinase protein was visualized at 24 kDa on SDS-PAGE. On heat treatment at 90°C for 60 min, chitinase protein was stable throughout the treatment (Figure 2a) and was visualized at 24 kDa, immunoblotting with chitinase antibodies showed that the immunoreactivity of the protein was also intact on heat treatment (Figure 2b).

3.4 Simulated gastric fluid (SGF) digestion: The digestibility of purified chitinase protein was examined in SGF for 60 mins. After, an hour of SGF incubation, chitinase protein was detectable on SDS-PAGE without any degradation (Figure 3). This indicated that chitinase was stable in SGF.

3.5 Specific IgE binding: The specific IgE binding to rice chitinase was assessed by ELISA using patients' sera (SPT positive to different food extracts). A total of 110 patients' sera were screened against chitinase protein and of these, seven serum samples (n=7) showed significant IgE binding to chitinase protein (Table 1). Sera from healthy subjects (n=3) were used as a negative control. OD values 3 times that of the control sera (OD of 0.089) were taken as the cutoff for an ELISA-positive result (Table 1).

3.6 Potency of the hypoallergenic mutants: Allergenic potential of chitinase protein was elucidated by ELISA inhibition assay. Chitinase positive pooled patients' sera (n=7), pre-incubated with graded amounts of chitinase, was employed for the inhibition assay. For chitinase the effective concentration required for 50% inhibition (EC_{50}) was 5.4ng (Figure S4).

3.7 Prediction of IgE binding epitopes of chitinase: Linear and conformational B-cell epitopes were predicted for rice chitinase and three consensus regions were identified as major IgE binding epitopes -

3.7.1 B-cell epitope prediction - Linear epitopes: Seven linear antigenic determinants were predicted for rice chitinase by most of the *in silico* tools used in the study. These regions are - A: 15 - 25, B: 31 - 47, C: 51 - 60, D: 91 - 106, E: 109 - 124, F: 135 - 147 and G: 148 - 166 (Supplementary Table 2).

3.7.2 3D homology based model was generated for *O. sativa* chitinase using Modeler 9.11v server: Jack bean chitinase structure (PDB: 1DXJ_A) served as template with sequence identity of 64% with rice chitinase. Query and the template protein were successfully aligned using Clustal W and 400 models were generated with the software. The model with the lowest DOPE score was selected for further studies. The stereo chemical quality of the model was assessed using PROCHECK, PROSA and Errat. PROCHECK for the model with minimum DOPE score showed that 89.5% of the main chain residues were in the most favorable region of the Ramachandran plot. None of the residues were present in the disallowed regions of the Φ and Ψ values. One loop region from 162 - 167, exceeded 99% error value in Errat. This region was optimized by loop refinement function of Modeler 9.11v software. 100 more models were

generated and the model with the minimum DOPE score was selected. The energy of the refined model was minimized using AMBER force field implemented in Deep View/Swiss PDB Viewer v4.1.0. The overall quality factor of 98.80% was assigned to chitinase in Errat (Supplementary Figure 1a) and PROCHECK for the final model showed that 90.2% of the main chain residues were in the most favorable region of the Ramachandran plot (Supplementary Figure 1b). Final structure was visualized using Jmol (<http://www.jmol.org/>) software **and the epitopic regions have been highlighted in the spacefill model of rice chitinase (Supplementary Figure 1 c, d).**

3.7.3 Conformational epitopes: Homology modeled 3D structure of rice chitinase was submitted to Disco Tope 2.0 Server and CBTOPE web servers for prediction of conformational epitopes (**Supplementary Table 5**). For selection of consensus epitopes i.e. overlapping linear and conformational epitopes, a preference was given to residues predicted by tools having an area under receiver operating characteristic curve (AUC) greater than 0.70. Regions recognized by three or more tools with default settings were selected. Three stretches of amino acids RC1 (12 - 25), RC2 (31 - 45) and RC3 (140 - 166) were recognized by at least four tools (Table 2). Antigenic index plot was obtained for rice chitinase using DNASTar program (Supplementary Figure 2).

3.8 IgE binding of the peptides: Synthetic peptides representing the epitopes were tested for IgE binding by ELISA using individual food allergic patients' sera (Figure 4). The cut off value for ELISA positive was taken as three times of the control value (OD 0.088). Peptide RC2 (epitope RC2) showed maximum IgE binding with 7/7 individual patients' sera. While peptide RC1 showed significantly higher IgE binding as compared to control but lower IgE binding

compared to chitinase. Individual sera from healthy volunteers (n=7) showed negligible binding with chitinase and the peptides in comparison to patients' sera.

3.9 Peptide similarity search of the predicted linear epitopes - Seven linear epitopes predicted for rice chitinase shared similar physicochemical properties with the sequences available in SDAP. These sequences were identified by calculating property distance (PD) values. Sequences with a PD value of less than 7 and high similarity were included in the study. Linear epitopes of rice chitinase D: 91 - 106, E: 109 - 124 and G: 148 - 166, displayed low PD value with chitinases from *Hevea brasiliensis*, *Persea americana* and *Castanea sativa* allergens (Supplementary Table 3). These values suggest that the allergen in question may share clinically significant IgE cross reactivity with food allergens due to similarity in the primary structure.

4. DISCUSSION

Rice is consumed in many countries across the globe, while in most of Asia it is consumed as a staple food crop. Allergy to rice is predominantly high in Southeast Asian countries like Japan and India, where the consumption is high compared to Europe and US (Wakasa, Hirano, Urisu, Matsuda, & Takaiwa, 2011). Chitinases from rice have been isolated and used as transgenic proteins for development of genetically modified varieties, due to antifungal properties. However, the allergenic potential needs to be evaluated before using it as a transgene. Present study, evaluates the allergenicity features associated with rice chitinase by *in silico* and immunobiochemical methods. Further, linear and conformational B cell epitopes for chitinase were predicted by different sequence and structure based tools.

Rice chitinase gene was cloned in pET28a+ expression vector, expressed in BL21 *E. coli* cells and affinity purified. Recombinant proteins are better suited for characterization studies, as they display high yield and minimal batch to batch variation. Several food allergens have been produced in recombinant form for improved diagnosis and therapy (Lidholm, Ballmer-Weber, Mari, & Vieths, 2006).

Food allergens are primarily proteins and possess an intact 3D conformation which may play a role in determining allergenic properties. Some characteristics are attributed to common food allergens like thermal stability, resistance to gastric digestion, stable to pH change etc. (Lehrer, Ayuso, & Reese, 2002). Investigating the stability of proteins in gastrointestinal tract, helps in distinguishing food allergens from non allergens (Wickham, Faulks, & Mills, 2009). In the present study, digestibility of chitinase was examined in SGF. Rice chitinase remained stable for 60 mins on incubation with pepsin. Thermal processing involves disrupting the native 3D structure of the protein leading to loss of conformational epitopes, activation of new epitopes or improving accessibility of cryptic epitopes (Cabanillas et al., 2014). Therefore, a broad temperature range is taken in to consideration for heat treatment experiments i.e. from 25°C to 95°C for up to 60 minutes. In the study, chitinase was not only stable throughout the heat treatment but immunoreactivity of the protein was also retained.

Previous studies suggest that rice proteins are cross reactive with IgE binding proteins of corn, soybean and peanut (Lehrer et al., 1999). A high degree of cross-reactivity among rice and corn may be accounted for the fact that they both belong to Poaceae family. Similar to previous findings, in the present study, out of 110 patients' sera screened 7 were allergic to corn, mustard, rice and tomato. However, further studies are required to confirm the clinically important cross

reactivity. Effective concentration required for 50% inhibition (EC_{50}) was 5.4ng for chitinase as observed by ELISA inhibition.

Pathogenesis related proteins represent a highly conserved protein superfamily with allergenic properties (Van Loon, Rep, & Pieterse, 2006). Members of PR-4 (chitinases) group along with other families are responsible for the wide cross-reactivity in pollen, food and latex allergic patients (Hoffmann-Sommergruber, 2002). Previous studies indicate that broad cross-reactivity among proteins from different plant species is generally due to 3D structural conservation of specific domains. It is hypothesized that members of PR-protein families from related or distant plant species, display conserved epitopes mediating broad cross-reactivity, regardless of the amino acid sequence divergence (Jimenez-Lopez, Gachomo, Ariyo, Baba-Moussa, & Kotchoni, 2011). Bioinformatics provide a powerful tool in the post-genomic era to predict protein structures, functions and protein–protein or protein–ligand interactions. A proper identification and characterization of the specific B and T cell epitopes is therefore critical for efficient clinical trials, allergy diagnosis and immunotherapy.

In the present study, a computational biology approach was used to identify B cell epitopes of rice chitinase and three regions were predicted as probable antibody binding regions. Synthetic peptides corresponding to these regions were commercially obtained to study the IgE binding potential of the identified B cell epitopes. Peptides RC1 & RC2 (epitope RC1 & RC2) both bind to the IgE of individual patients' sera positive to chitinase but the binding efficiency varies. RC2 showed high IgE binding with 7/7 patients' sera, whereas RC1 showed low binding compared to RC2 but higher than that of control (Figure 4). These findings can further be understood by developing hypoallergenic variants corresponding to individual epitopes and further estimating the IgE binding levels of the hypoallergenic variants to that of chitinase.

However, ELISA findings suggest that the *in silico* predicted regions may act as probable B cell epitopes of rice chitinase.

Computational approach for epitope prediction is less time consuming compared to conventional methods (Mishra, Jain, & Arora, 2015), but *in silico* predicted regions should be validated experimentally. Sequence homology studies for chitinase have shown, it shares identity with *Hevea brasiliensis*, *Persea americana* and *Castanea sativa* allergens (Mishra, Gaur, Singh, & Arora, 2012). These findings are similar to PD similarity index values calculated for linear epitopes of rice chitinase. The selection of the PD value cut off was such, where a low value was selected as threshold (<7) in order to evict less similar sequences. These findings suggest that identified epitopes which give hit with SDAP allergens may share cross reactivity with some of the known food allergens (Maleki et al., 2011). **However, further studies are required to confirm the structural integrity of the recombinant rice chitinase by circular dichroism (CD).**

In conclusion, the present study identified three potential B cell epitopes for rice chitinase using a combination of bioinformatics tools. The structural and physio-chemical properties associated with IgE binding were evaluated and chitinase was identified as a potential allergen. **However, more information needs to be gathered from ELISA inhibition studies for predicting clinically important cross reactivity of the predicted B cell epitopes.** And similar approach may be employed for prediction of epitopes in novel proteins.

Acknowledgements: AM is a Senior Research Fellow (SRF) of Indian Council of Medical Research (ICMR), New Delhi.

Conflict of interest: Authors have no financial or any kind of non-financial conflict of interests.

Author contributions: Conceived and designed the experiments: AM & NA. Performed the experiments: AM. Analyzed the data: AM & NA. Contributed reagents/materials/analysis tools: SNG, SL & NA. Wrote the paper: AM & NA.

Note:

- The recombinant plasmid containing chitinase gene²⁸ was purchased from BIOGENE Co. And later the gene was sub-cloned in an expression vector i.e. pET28a(+). Recombinant chitinase protein was purified with the N-terminal 6 x His tag of the vector.
- Peptide for third epitope i.e. epitope RC3 could not be synthesized by the manufacturer. Due to technical issues with the synthesis process. Therefore, epitope RC3 (peptide) was not used for IgE binding studies.

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Figure Legends:

Figure 1: Purified chitinase protein resolved on 12% SDS – PAGE **under reducing conditions**.

Lane MW: molecular weight protein ladder; Lane A: Coomassie brilliant blue stained chitinase protein (**18µg**); Lane B, C & D: Immunoblot with - pooled patients' sera, mice raised antibodies against chitinase & **Anti His-tag antibody**, respectively. **And the amount of protein loaded in Lane B, C & D was 15 µg.**

Figure 2 (a): SDS – PAGE (**under reducing conditions**) profile of heat treated chitinase protein. Chitinase was heated for 0, 15, 30, 45 and 60 mins at 90°C and resolved on 12% gel stained with Coomassie brilliant blue. Lanes: MW, molecular weight protein ladder; 0 – 60 time in minutes. **Figure 2 (b):** Heat treated chitinase transferred on to nitrocellulose membrane and probed with chitinase antibodies, **0min – 60mins** different time intervals.

Figure 3: SDS – PAGE (**under reducing conditions**) profile of chitinase protein treated with SGF. Chitinase was treated with SGF for 0, 5, 15, 30, 45 and 60 mins and resolved on 12% gel, stained with Coomassie brilliant blue. Lane M: molecular weight protein ladder; Lane 1 – 7: SGF samples at different time intervals.

Figure 4: IgE binding of the predicted peptides was tested with 7 individual food allergic patients' sera: Recombinant chitinase, peptide RC1 or peptide RC2 were coated on ELISA plate and incubated with food allergic patients' sera. The IgE affinity was detected by HRP labeled anti human IgE and sera from 7 healthy subjects was taken as control. Peptides RC1 and RC2 showed increased IgE binding compared to control ($p < 0.001$). Peptide RC1 showed reduced IgE

binding compared to rice chitinase ($p < 0.001$). **Statistical significance is represented as (***)**
 $p < 0.001$ compared to rice chitinase.

Table 1: Specific IgE binding of chitinase was measured by ELISA using patients' sera. Out of 110 patients' sera screened, 1 - 7 samples showed IgE binding (ELISA) with chitinase. Control serum samples 8 - 10 were from healthy individuals.

S. No.	Allergen (History, SPT Positive)	Specific IgE(OD 492 nm)	
		Chitinase	Corresponding allergen
1	Corn	0.427	0.459
2	Mustard	0.441	0.448
3	Rice	0.408	0.427
4	Corn	0.358	0.368
5	Rice	0.366	0.367
6	Mustard	0.376	0.388
7	Tomato + Mustard	0.392	0.451
8	Control	0.114	-
9	Control	0.089	-
10	Control	0.098	-

Table 2: On comparing predicted linear and conformational epitopes the consensus regions are:-

B - cell Epitopes	Sequence	Position	No. of Residues
RC1	ETTGGTRGSSDQFQ	12 – 25	14
RC2	KEEINKATSPPYGR	31 – 45	15
RC3	NDANVDRIGYYKRYCDMLGTGYGSNLD	140 – 166	27

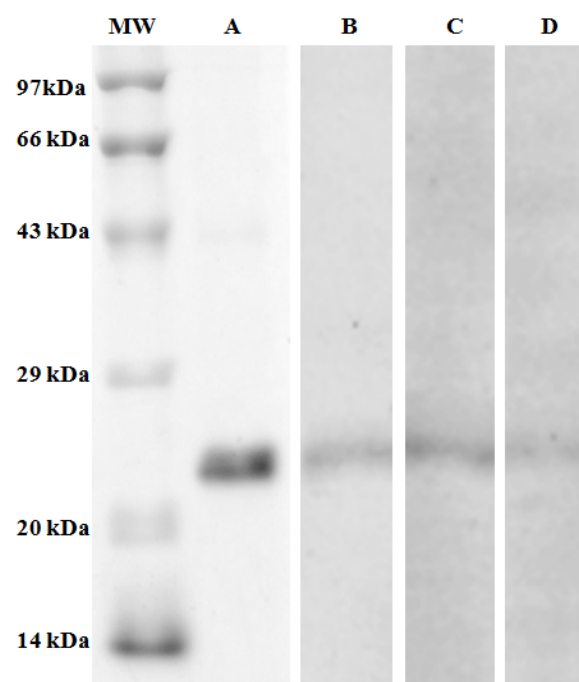
Figure 1

Figure 2 (a)

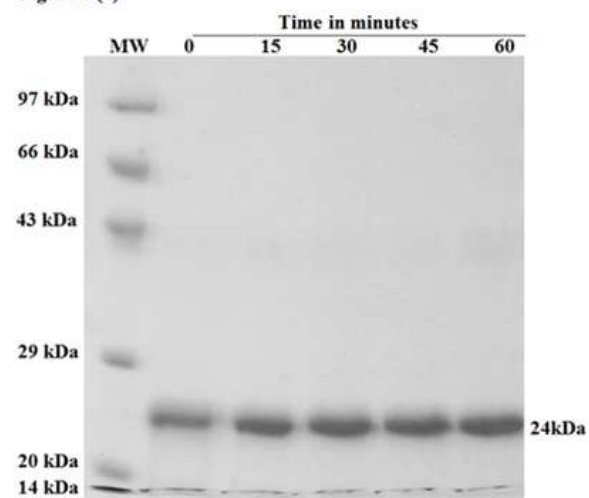
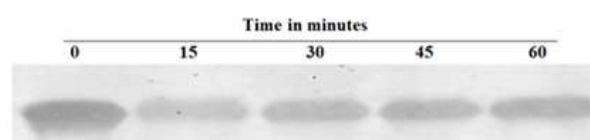


Figure 2 (b)



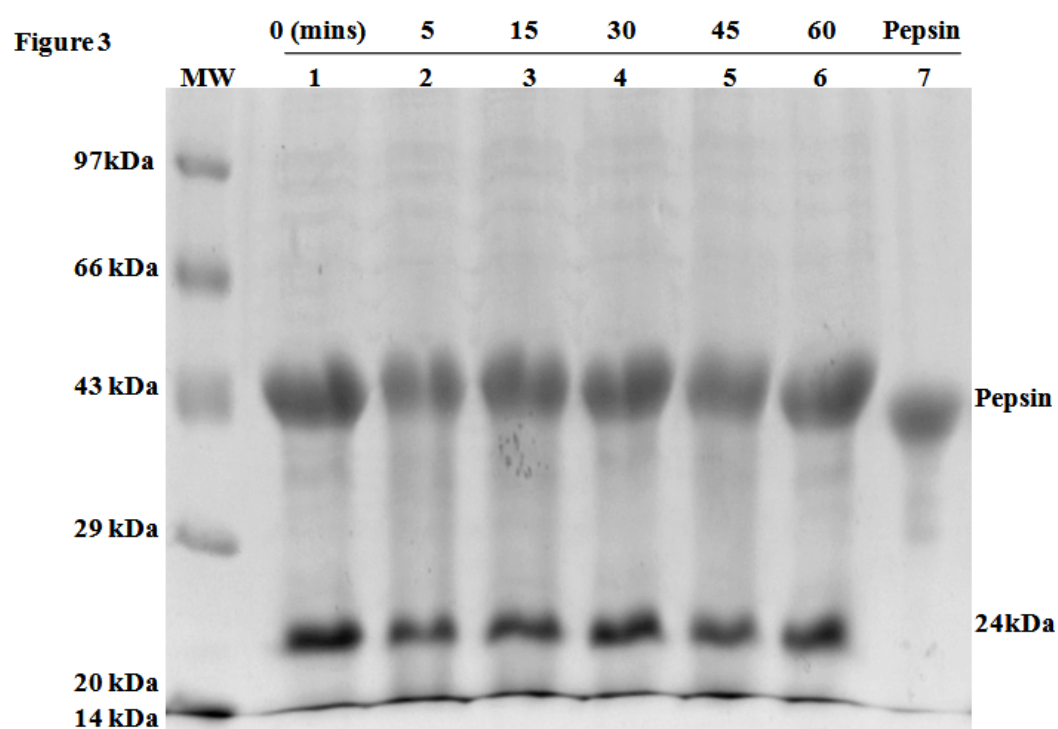
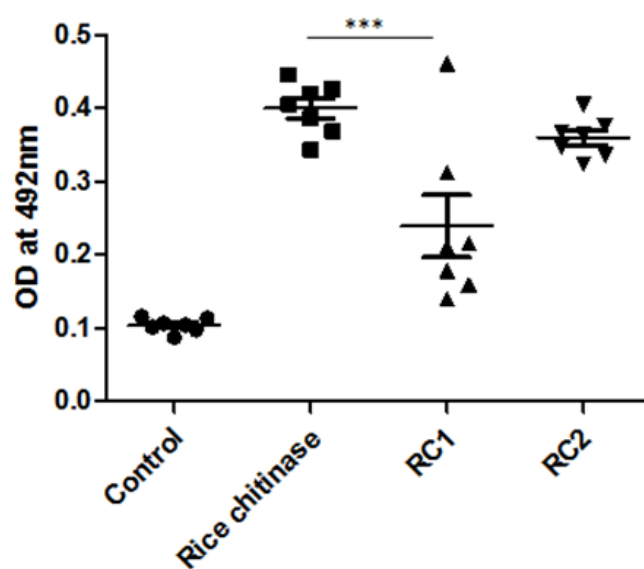


Figure 4



***: $p < 0.001$ represents statistical significance compared to rice chitinase

Highlights:-

- Expression and purification of rice chitinase protein for immunobiochemical studies
- 3D structure of rice chitinase was generated by homology modeling
- *In silico* identification of B-cell epitopes of rice chitinase
- Predicted B-cell epitopes were validated by *in-vitro* IgE binding studies
- Rice chitinase identified as a potential allergen by *in silico* and *in vitro* studies

Supplementary Figure Legends:

Figure S1 (a): Error values for residues as predicted by ERRAT for chitinase structure: Y axis presents the error value and X axis presents the amino acid sequence of chitinase. The overall quality factor assigned to chitinase model (Rcht2.BL00240001) was 98.80%. **Figure S1 (b):** Ramachandran plot for the amino acid residues in the *Oryza sativa* chitinase model: Stereochemical quality of the 3D model analyzed by PROCHECK. 90.2 % of residues were present in most favourable region, 9.8% in additional allowed region and none in generously allowed or disallowed regions. **Figure S1 (c):** Three dimensional structure of *O. sativa* chitinase visualized using Jmol. Region in green is the loop refined part of the model. **Figure S1 (d):** Three dimensional structure of *O. sativa* chitinase visualized using Jmol. The complete structure is represented as a spacefill model in grey colour. All the three epitopes are marked in different colours - Epitope 1 (RC1: 12 - 25) in magenta, Epitope 2 (RC2: 31 - 45) in yellow and epitope 3 (RC3: 140 - 166) in cyan.

Figure S2: Antigenic index profile of rice chitinase by DNASTAR protean system. Grey panels depict *in silico* predicted B-cell epitopic regions on rice chitinase sequence.

Figure S3: Full length FASTA sequence alignment of rice chitinase with *Persea americana* Pers a 1 (Figure S3a), *Musa acuminata* Mus a 2 (Figure S3b) *Hevea brasiliensis* Hev b 11 (Figure S3c), & *Castanea sativa* Cas s 5 (Figure S3d). Epitopes corresponding to the predicted peptides have been highlighted in yellow. And linear epitopes D, E & G have been marked in blue. This figure has been uploaded as a pdf to maintain clarity.

Figure S4: ELISA inhibition for evaluating the allergenic potency of rice chitinase. For IgE inhibition, chitinase positive patients' pooled serum was incubated with 0.1-1000ng of chitinase protein as a self inhibitor. The assay was carried out with purified chitinase protein coated on a microtiter plate.

Figure S1a

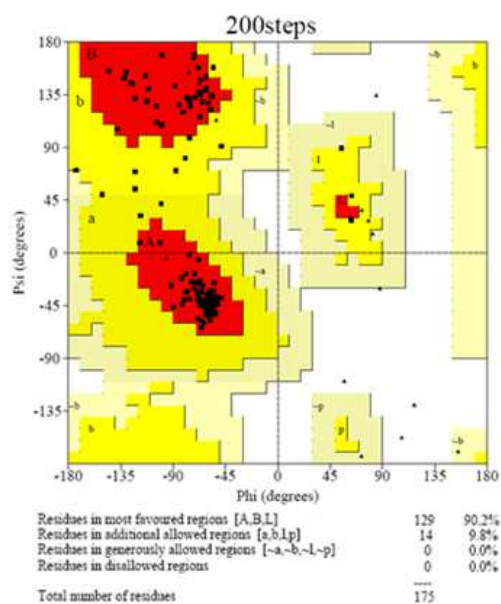


Figure S1b

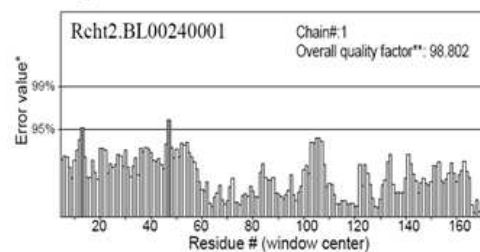


Figure S1c



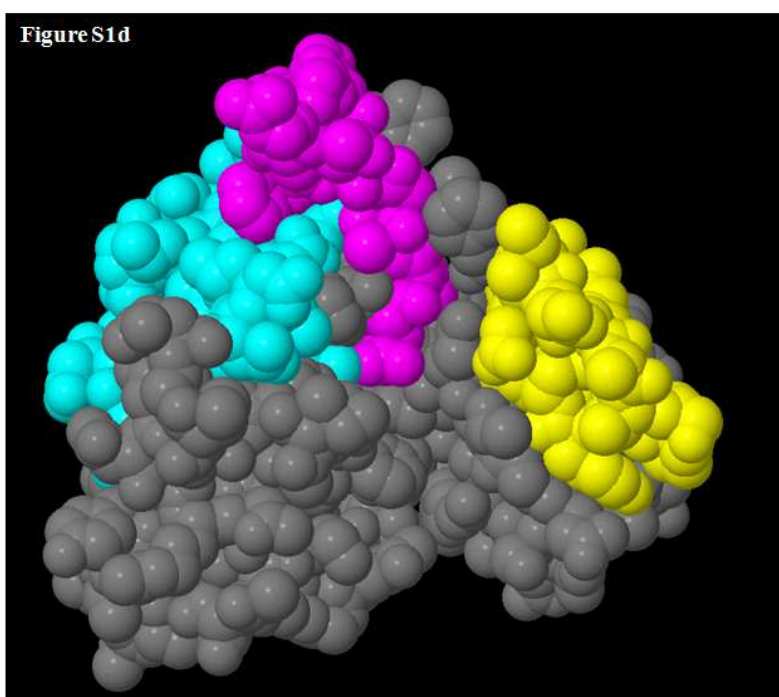


Figure S2



Alignment made with [FASTA 3.45](#)

Sequence 1: UserSequence

Sequence 2: Allergen [Pers a 1](#), Sequence: [CAB01591](#)

Sequence identity: 67.43 (118/175)

```

Sequence 1 -----
Sequence 2 MVYCTASLPLLLLLLVGLLAGEAFAEQCGRQAGGALCPGGLCCSQFGWCG
Sequence 1 -----
Sequence 2 STSDYCGPTCQSQCGGVTPSPGGGVASLISQSVFNQMLKHRNDAACQAKG
Sequence 1 -----ELAAFFGQTSHETTGGTRGSSD
Sequence 2 FYTYNAFIAAANSFNGFASVGDTATRKREIAAFLAQTSHETTGGWATAPD
Sequence 1 -QFQWGYCF-KEEINK-----ATSPPPYYGRGPIQLTGQSNYQA
Sequence 2 GPYAWGYCFLKEQGNPPDYCVPTAQWPCAPGKKYYGRGPIQISYNYNYGP
Sequence 1 AGNALGLDLVGNPDLVSTDAVVSFKTAIWFWMTAQGNKPSCHDVILGRWT
Sequence 2 AGRAIGYDLINNPDAVATDPVISFKTALWFWMTQSPKPSCHNVITGRWT
Sequence 1 PSAADTAAYRVPGYDGITNIINGGIECGVGQNDANVDRIIGYYKRYCDMLG
Sequence 2 PSAADAAARPGY--ITNIINGGIECG-G-ND---DRIG-YKRYCD-LG
Sequence 1 TGYGSNLDCYNQRFAS-----
Sequence 2 VSYGSNLDCYNQRSFGVSTNPLAASS

```

Sequence 1: UserSequence

Sequence 2: Allergen [Cas s 5](#), Sequence: [Q42428](#)

Sequence identity: 67.43 (118/175)

Sequence 1 -----

```

-----
Sequence 2 MKLFSLLLFLAFLLGTSAEQCGRQAGGAACANNLCCSQFGWCGNTAEYCG
Sequence 1 -----
-----
Sequence 2 AGCQSQCSSPTTTTSSPTASSGGGGDVGSLISASLFDQMLKYRNDPRCKS
Sequence 1 -----ELAAFFGQTSHETTGGTRGS
-----ELAAF--QTSHETTGG----
Sequence 2 NGFYTYNAFIAAARSFNGFGTTGDVTTTRKRELAFLAQTSHETTGGWATA
Sequence 1 SD-QFQWGYCFKEEINKAT-----SPP-----YYGRGPIQLTGQSNYQA
-D----WGYCF--E-NK-T-----S-P-----YYGRGPIQLT---NY--
Sequence 2 PDGPYAWGYCFVMENNKQTYCTSKSWPCVFGKQYYGRGPIQLTHNINYGO
Sequence 1 AGNALGLDLVGNPDLVSTDAVVSFKTAIWFWMTAQGNKPSCHDVILGRWT
AG-A-G-DL--NPDLV-T----SFKTAIWFWMT-Q-NKPS-HDVI-G-W-
Sequence 2 AGKAIGADLINNPDLVATNPTISFKTAIWFWMT-PQANKPSSHDVIIGNWR
Sequence 1 PSAADTAAYRVPGYDGITNIINGGIECGVGQNDANVDRI GYYKRYCDMLG
PSAADT-A-RVP-Y--ITNIINGG-ECG-G--D----RIG-YKRYCD-LG
Sequence 2 PSAADTSAGRVPSYGVITNIINGGLECGHGSDDRANRIGFYKRYCDTLG
Sequence 1 TGYGSNLDCYNQRFAS
--YG-NLDCYNQ--FA-
Sequence 2 VSYGNNLDCYNQKPFA-

```

Sequence 1: UserSequence

Sequence 2: Allergen [Mus a 2.0101](#), Sequence: [Q8VXF1](#)

Sequence identity: 65.14 (114/175)

```

Sequence 1 -----
-----
Sequence 2 MKALLLVIFTLASSLGAFAEQCGRQAGGALCPGGLCCSQYGWCGNTDPYC
Sequence 1 -----

```

```

Sequence 2 -----
GQGCQSQC GSGSGGSGVASIISSSLFEQMLKHRNDAACPGKGFYTYNA

Sequence 1 -----ELAAFFGQTSHETTGGTRGSSD-QFQWG
-----E-AAF--QTSHETTGG-----D----WG
Sequence 2 FIAAANSFSGFGTTGDDAKKKREIAAFLAQTSHETTGGWATAPDGPYAWG

Sequence 1 YCFKEEINK-----ATSPPYYGRGPIQLTGQSNYQAAGNALG
YCF--E-N-----A----YYGRGPIQ-----NY--AG-A-G
Sequence 2 YCFVQEQNPSSDYCVASSQWPCAAGKKYYGRGPIQISFNINYGPAGRAIG

Sequence 1 LDLVGNPDLVSTDVVSFKTAIWFWMTAQGNKPSCHDVILGRWTPSAADT
-DL--NPDLV-TDA--SFKTA-WFWMT-Q--KPSCHDVI-G-WTPS-AD-
Sequence 2 SDLLNNPDLVATDATISFKTALWFWMTQSPKPSCHDVITGSWTPSNADQ

Sequence 1 AAYRVPGYDGIITNIINGGIECGVGQNDANVDRI GYYKRYCDMLGTGYGSN
AA-R-PGY---TNIINGG-ECG-G-----DRIG-YKRYCD-LG--YG-N
Sequence 2 AAGRLPGYGVTTNIINGGLECGKGYDARVADRIGFYKRYCDLLGVSYGDN

Sequence 1 LDCYNQRNFAS-----
LDCYNQR-FAS-----
Sequence 2 LDCYNQRPFASTAATATF

```

Sequence 1: UserSequence

Sequence 2: Allergen [Hev b 11.0102](#), Sequence: [CAD24068](#)

Sequence identity: 68.57 (120/175)

```

Sequence 1 -----
-----
Sequence 2 EQCGRQAGGALCPGGLCCSQYGWcantPEYCGSGCQSQC DGGVGGEGGCV

Sequence 1 -----
-----
Sequence 2 DLGSIISRSTFEEMLKHRNNAACPAKGFYTYDAFISAAKAFPAFGTTGDV

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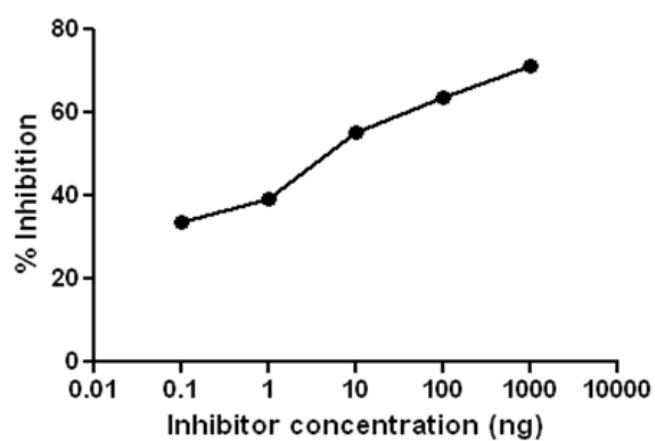
Sequence 1 -----ELAAFFGQTSH**ETTGGTRGSSD-QFQ**WGYCF**KKEEINKATS**---**P**-
 -----E-AAFFGQTSH-TTGG-----D-----WGYC-KEE-N-A-S---P-
 Sequence 2 DTCKREIAAFFGQTSHATTGGWPTAPDGPYAWGYCHKEELNQASSYCSPS

Sequence 1 **P**-----**YYGR**GPIQLTGQSNYQAAGNALGLDLVGNPDLVSTDAVVS
 P-----YYGRGPIQL----NY---G-ALGLDL--NPDLV-TD-V-S
 Sequence 2 PAYPCAPGKKYYGRGPIQLSWNYNYGQCGQALGLDLLNPNPDLVATDRVIS

Sequence 1 FKTAIWFWM**TAQGNKPSCHDVILGRWT****PSAADTAAYRVPGYDGI**TNIING
 FK-AIWFWM-T-Q--KPSCHDVI-G-W-P---D--A-R-PGY--ITNIING
 Sequence 2 FKAAIWFWMTPQFPKPSCHDVITGQWSPTGHDISAGRAPGYGVITNIING

Sequence 1 GIECGVG**QNDANV-DRIG**YYKRYCD**MLGTGYGSNLD**CYNQRNFAS-
 G-ECG-G--DA-V-DRIG-YKRYCDM-G-GYGSNLD CYNQ--F---
 Sequence 2 GLECGSGW-DARVEDRIGFYKRYCDMFGVGYGSNLD CYNQTPFGLG

Figure S4



ELISA Inhibition

Supplementary Table 1: Synthetic peptides corresponding to the predicted B cell epitopes

Peptides	Sequence	No. of residues	% purity
RC1	ETTGGTRGSSDQFQ	14	>95%
RC2	KEEINKATSPPYGR	15	>95%
RC3	NDANVDRIGYYKRYCDMLGTGYGSNLD	27	-

Supplementary Table 2: Linear IgE binding epitopes predicted by online web servers

S. No.	Predicted B-cell epitopes	Position	Prediction servers
A	GGTRGSSDQFQ	15 - 25	ABCpred BcePred BepiPred 1.0 Server DNASTAR Lasergene version 7.2 BCPreds Server 1.0
B	KEEINKATSPPYGRGP	31 - 47	
C	TGQSNYQAAG	51 - 60	
D	TAQGNKPSCHDVILGR	91 - 106	
E	PSAADTAAYRVP GYDG	109 - 124	
F	CGVGQNDANVDRI	135 - 147	
G	GYYKRYCDMLGTGYGSNLD	148 - 166	

Supplementary Table 3: Peptide similarity search of the three predicted epitopes of chitinase with known allergens in SDAP database. The table shows similarity of the predicted B- cell epitopes of chitinase with peptides of known allergens with a PD value less than 7.0 in SDAP database.

Allergen	PD Seq. similarity index
(Linear Epitope D): TAQGNKPSCHDVILGR	
<i>Persea americana</i> Pers a 1	4.41
<i>Castanea sativa</i> Cas s 5	5.09
<i>Musa acuminata</i> Mus a 2.0101	5.14
<i>Hevea brasiliensis</i> Hev b 11.0101	5.77
(Linear Epitope E): PSAADTAAYRVPGYDG	
<i>Persea americana</i> Pers a 1	5.91
<i>Castanea sativa</i> Cas s 5	5.99
<i>Musa acuminata</i> Mus a 2.0101	6.76
(Linear Epitope G): GYYKRYCDMLGTGYGSNLD	
<i>Hevea brasiliensis</i> Hev b 11.0101	2.00
<i>Persea americana</i> Pers a 1	2.64
<i>Musa acuminata</i> Mus a 2.0101	3.27
<i>Castanea sativa</i> Cas s 5	3.44

Supplementary Table 4: Rice chitinase gene share sequence identity with the allergens listed in three databases namely – SDAP, Allermatch and FARRP.

Allergens	Full length FASTA alignment		80 aa sliding window - sequence identity %
	% Identity	E-value	
SDAP			
<i>Persea americana</i> (Pers a 1)	67.43 (118/175)	2.8e-47	76.25
<i>Musa acuminata</i> (Mus a 2.0101)	65.14 (114/175)	1.7e-45	61.25
<i>Hevea brasiliensis</i> (Hev b 11.0102)	68.57 (120/175)	2.2e-44	72.50
FARRP			
<i>Persea Americana</i> (Endochitinase)	63.10	4.2e-045	76.25
<i>Castanea sativa</i> (chitinase Ib)	63.40	1.8e-043	71.30
<i>Hevea brasiliensis</i> (class I chitinase)	63.80	2.5e-042	70.40

Supplementary Table 5: Conformational IgE binding epitopes predicted by online web servers.

S. No.	Predicted B-cell epitopes	Position	Prediction servers
1	ETTGGTRGS	12 - 20	Disco Tope 2.0 Server CBTOPE web server
2	KATSPPYYG	36 - 44	
3	NDANVDRI	140 - 147	
4	RYCDMLGTGY	152 - 161	