



# Immunogenic peptides: B & T Cell Epitopes of Per a 10 Allergen of *Periplaneta americana*



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## ABSTRACT

Mapping of B and T cell epitopes of an allergen can be utilised in the development of alternative therapeutic modalities and diagnostics. The present study was aimed to identify B and T cell epitopes of Per a 10, a major cockroach allergen, by computational tools and subsequent validation by *in vitro* experiments. Per a 10 three-dimensional structure was homology modelled using structure of anionic trypsin from pacific chum salmon as a template. Seven B cell epitopes (B-P1 to B-P7) were predicted by sequence and structure based methods. Three T cell epitopes (T-P8 to T-P10) were predicted by binding score and inhibitory concentration dependent prediction tools. Predicted epitopes were synthesized and biological activity was assessed by ELISA, ELISA inhibition and PBMC proliferation assays. B cell peptides B-P5, B-P6 and B-P7 showed significantly high IgE binding with pooled and individual cockroach hypersensitive patients' sera while the T cell peptides did not show IgE binding. ELISA inhibition was performed to determine the potency of the predicted peptides. Fifty nanogram of peptide B-P7 was required for 50% IgE binding inhibition of surface bound Per a 10 whereas seventy five nanogram and ninety nanogram of B-P5 and B-P6 were required for the same respectively. Upon stimulation with T-P8 and T-P10 peptides, PBMCs from cockroach allergic patients' (n = 15) showed significant lymphocyte proliferation and induced IL-4 and IL-5 cytokine release in the culture supernatant demonstrating Th2 dominant cell mediated response of predicted T cell peptides. In conclusion, Per a 10 3-D structure obtained by homology modelling was used to identify B and T cell epitopes, followed by *in vitro* validation. The identified peptides can be potentially used in designing diagnostics and therapies for cockroach allergy.

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## 1. Introduction

Airway allergies are a major contributor to the pathogenesis of asthma and exposure to indoor allergens (such as house dust mite, insects, cat, rodent and fungal spores) is considered as a major risk factor for the same (Lambrecht and Hammad, 2014; Pawankar et al., 2014 and Arruda et al., 2001). Childhood exposure to cockroaches has been associated with asthma and increased bronchial hyper-responsiveness (Rosenstreich et al., 1997; Platts-Mills et al., 2000).

**Abbreviations:** HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell; DOPE, discrete optimized protein energy; NHS, normal human sera; ADH, alcohol dehydrogenase; SDAP, structural database of allergenic protein.

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*Blattella germanica* and *Periplaneta americana* are two domiciliary cockroach species predominating in temperate and tropical regions respectively (Sookrung and Chaicumpa, 2010). *Periplaneta americana* consists of 22 IgE binding proteins, of which nine proteins have been characterised as allergens (Sudha et al., 2008; Pomés et al., 2007; Fang et al., 2015). Nine allergens have been characterised from *Blattella germanica* as well (Pomés et al., 2007; Jeong et al., 2013).

Proteases from a variety of allergens such as *Curvularia lunata*, *Aspergillus fumigatus*, *Penicillium chrysogenum* and *Dermatophagoides pteronyssinus* have been characterised as potent allergens (Sharma et al., 2009; Shen et al., 2007; Kinga et al., 1996). Per a 10, a major serine protease allergen was found to react with 85% of cockroach hypersensitive patients' sera (Sudha et al., 2008). Recombinant Per a 10 (rPer a 10) has also been characterised and its allergenic potential was found to be lower than the native Per a 10 (nPer a 10) protein (Govindaraj et al., 2013). Per a 10 drives airway

allergic responses and acts as an adjuvant for the bystander allergen ovalbumin by virtue of its serine protease activity (Sudha et al., 2009). It activates PAR-2 (Protease activated receptor-2) on airway epithelial cells and modulates dendritic cell response towards Th2 by upregulation of CD86 and OX40L, IL-23 production, CD40 cleavage and reduced IL-12p70 levels (Goel et al., 2012; Agrawal et al., 2015).

Identification of three-dimensional structure of allergen and epitopes can be used in the better understanding of immune response to allergenic proteins, including the cross-reactivity among homologous allergens and designing of hypoallergenic variants of allergens and peptides for immunotherapy (Pomés, 2010). B and T cell epitopes exhibit fundamental differences in their localisation on allergen molecule as well as in eliciting the immunological responses (Pomés, 2010). B cell epitopes can either be linear or conformational and are usually located on the surface of the allergen thereby binding readily with antibody molecules. T cell epitopes are linear antigenic peptide sequences distributed throughout the primary structure of allergen (Pomés, 2010). They are presented in the context of MHC II on the surface of antigen presenting cells (APCs) and are recognised by specific T cells (Galli et al., 2008). Several methods are available to identify the epitopes, such as X-ray crystallography, enzymatic fragmentation, overlapping synthetic peptides, recombinant fragments of the allergen, epitope expression, cDNA libraries, digested allergens and site directed mutagenesis (Frazier et al., 2014).

*In-silico* epitope prediction relies on computational algorithms and offers certain advantages over the traditional approaches. These methods are rapid, scalable, fairly accurate and cost effective. An increase in the number of databases and *in-silico* tools have greatly aided in the identification of epitopes from numerous allergens (Sharma et al., 2009; Nair et al., 2011; Mishra et al., 2016; Li et al., 2014). Epitope mapping paves way for the development of alternative therapies and diagnosis of allergies (Larché, 2007). Computationally predicted epitopes demonstrate potential for generation of hypoallergenic variants by mutating B cell epitopes and peptide immunotherapy employing T cell epitopes (Sharma et al., 2013; Pomés and Arruda, 2014). Hypoallergenic variants of allergen can be engineered by mutating critical amino acids in IgE binding epitopes. In a study, predicted IgE binding epitopes of osmotin, a stress-responsive protein from *Nicotina tabacum*, were utilised in the development of hypoallergenic mutant protein with 90% reduction in IgE binding (Sharma et al., 2013). Likewise, T cell peptides can be used in immunotherapy and offer a safer alternative over crude allergen immunotherapy (AIT) (Larché, 2007). Immunotherapy with overlapping T cell epitope peptides of bee venom phospholipase A2 has been efficacious (Müller et al., 1998). Immunotherapeutic potential of *in-silico* predicted T cell epitopes has been validated by *in vitro* assays for Cur I 3 fungal allergen (Sharma et al., 2009).

Identification of B and T cell epitopes of Per a 10 allergen will be useful in understanding the allergen antibody responses as well as aid in the development of new diagnostics and therapy regimens for cockroach allergy. Therefore, the present study was undertaken to determine the molecular structure of Per a 10 and *in-silico* prediction of B cell and T cell epitopes followed by their validation.

## 2. Materials and methods

### 2.1. Homology Modelling of Per a 10

#### 2.1.1. Template selection

Per a 10 sequence (NCBI accession no. AY792954) was retrieved from NCBI and homology searched in the Protein Data Bank (PDB). The homologous template suitable for Per a 10 was selected by

BLAST server (<http://ncbi.nlm.nih.gov/>). The crystal structure of template was retrieved from PDB and further used for homology modelling. Secondary structures were predicted by PSIPRED online tool (Buchan et al., 2013).

#### 2.1.2. Sequence alignment

Amino acid sequence of target Per a 10 and template protein were pairwise aligned using ClustalW multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/clustalw/>) with default parameters. The alignment data file was used for homology modelling of Per a 10.

#### 2.1.3. Homology modelling

Per a 10 structure was modelled based on the crystal structure of anionic trypsin from pacific chum salmon (PDB ID: 1mbq). Four hundred protein models were generated by using the academic version of MODELLER 9v11 (Sánchez and Sali, 2000) and evaluated using Discrete Optimized Protein Energy (DOPE) assessment function to select the most suitable model structure. Stereochemical quality of the selected model was evaluated using SAVES (<http://nihserver.mbi.ucla.edu/SAVES.3/saves.php>) server which includes ERRAT, PROCHECK and Verify 3D online tools. Further, the incorrectly modelled loop regions were identified and refined using MODELLER 9v11. The final model was optimized by energy minimization using AMBER force field and steepest descent algorithm of the SYBYL 8.0 software from Tripos Inc. (St. Louis, MO). The stereochemical quality of the model was evaluated again by the above mentioned tools. The Per a 10 model was superimposed onto template crystal structure in Discovery Studio Visualizer 3.0 software to assess the similarity in tertiary structure.

### 2.2. Prediction of B cell epitopes

The B cell epitopes were predicted based on the physico-chemical characteristics of amino acids, like hydrophobicity, surface accessibility, antigenicity, and pocket characteristics (Sharma et al., 2009; Nair et al., 2011 and Sharma et al., 2013). For determination of linear epitopes, the sequence of Per a 10 was submitted to several prediction softwares namely ABCpred, Antigenic, BepiPred 1.0b, Bcepred, BCPREDS, DNaSTAR, ElliPro and Epitopia. Discontinuous epitopes were predicted by submitting the homology modelled structure to Conformational Epitope Prediction server (CEP) and DiscoTope. While taking the consensus of epitopes generated by various softwares, preference was given to results predicted from tools tested on larger datasets and having an area under receiver operating characteristic curve (AUC) greater than 0.65. Least preference was given to tools which solely rely on physico-chemical parameters. The regions recognised frequently by five or more than five tools were selected.

### 2.3. Prediction of T cell epitopes

T cell epitopes of Per a 10 were predicted by inhibitory concentration (IC<sub>50</sub>) value based quantitative prediction methods namely MHCpred (<http://www.ddg-pharmfac.net/mhcpred/MHCPred/>), SVMHC (<http://svrmhc.biolead.org/>) and SMM-Align ([http://tools.immuneepitope.org/analyze/html/mhc\\_processing.html](http://tools.immuneepitope.org/analyze/html/mhc_processing.html)). Online tools namely MULTIPRED, ProPred (<http://www.imtech.res.in/raghava/propred/>), RANKPEP (<http://bio.dfci.harvard.edu/RANKPEP/>) and SVMHC (<http://abi.inf.uni-tuebingen.de/Services/SVMHC>) which employ binding status scoring qualitative prediction methods were also used for T cell epitope prediction (Nair et al., 2011). Promiscuous peptides binding to multiple HLA class II molecules were selected as T cell epitopes. B and T cell epitopes recognised by computational tools were mapped onto

the homology modelled three dimensional structure of Per a 10 to determine their position and secondary structure.

#### 2.4. Patient sample

Allergic patients' (showing positive skin prick test to cockroach extract) from the Outpatient Department (V.P chest institute, Delhi) and from the allergy clinics/centres (Bengaluru Allergy Centre and US Lavasa Medical and Research Centre, Chandigarh) were recruited for the present study. Blood was withdrawn from patients' by trained professionals and was used for PBMC isolation and sera for IgE binding studies. The study protocol was approved by Institutional Human Ethics Committee and informed written consent was obtained from all participating subjects.

#### 2.5. Purification of Per a 10

*P. americana* allergen extract was prepared 1:50 (wt./vol) in phosphate-buffered saline (PBS, pH-7.4) with 10 mM phenyl-methylsulfonyl fluoride and 5 mM EDTA (Sigma Chemical Co., St. Louis, MO). The native Per a 10 protein was purified by affinity chromatography as previously described (Sudha et al., 2008).

#### 2.6. Peptide synthesis

The ten predicted epitopes (peptides) were chemically synthesized by Thermo fisher Scientific, Germany with a purity of  $\geq 95\%$  and the sequences were confirmed by mass spectrometry (MALDI-TOF) analysis.

#### 2.7. Enzyme linked immunosorbent assay (ELISA)

##### 2.7.1. ELISA with pooled cockroach hypersensitive patient sera

ELISA was performed with pooled patients' sera ( $n=6$ ). Briefly, the microtiter plates (Covalink Nunc TM Immunomodule, Roskilde, Denmark) were coated with  $1 \mu\text{g}/100 \mu\text{l}$ /well of cockroach extract (CE) and  $100 \text{ ng}/100 \mu\text{l}$ /well of Per a 10/B or T cell peptides/control peptide in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at  $4^\circ\text{C}$ . The plates were washed with PBS twice and then blocked with 3% defatted milk ( $200 \mu\text{l}$ /well) for 3 h at  $37^\circ\text{C}$ . After washing with PBS, the plates were incubated with  $100 \mu\text{l}$  of pooled cockroach hypersensitive patients' sera (1:10 v/v in PBS) at  $4^\circ\text{C}$  overnight. Post incubation the plates were washed with PBS-T (PBS containing 0.05% Tween 20) twice, followed by PBS twice. The plates were incubated with 1:1000 v/v diluted anti-human-IgE peroxidase (Sigma Chemicals Co., St. Louis, MO, USA) at  $37^\circ\text{C}$  for 2 h and washed with PBS-T twice followed by PBS thrice. The reaction was developed with *o*-phenylene diamine in citrate buffer (50 mM citric acid and 100 mM sodium phosphate) and stopped by adding  $50 \mu\text{l}$  of 5N  $\text{H}_2\text{SO}_4$  and absorbance was read as the optical density at 492 nm ( $\text{OD}_{492}$ ) in ELISA reader (Bio-Rad, Benchmark plus).

##### 2.7.2. ELISA with individual cockroach hypersensitive patient sera

Specific IgE ELISA was performed to determine the IgE binding of the B and T cell peptides. The microtiter plates (Covalink Nunc TM Immunomodule, Roskilde, Denmark) were coated with  $100 \text{ ng}/100 \mu\text{l}$ /well of Per a 10/B or T cell peptides in carbonate-bicarbonate buffer (pH 9.6). The plates were blocked with 3% defatted milk ( $200 \mu\text{l}$ /well) for 3 h at  $37^\circ\text{C}$  and washed with PBS and then incubated with  $100 \mu\text{l}$  of 10 individual cockroach hypersensitive patients' sera (1:10 v/v in PBS) at  $4^\circ\text{C}$  overnight. Sera from 5 healthy subjects without a history of allergic asthma and rhinitis were used as control normal human sera (NHS). After incubation the plates were processed as described in the above section and

absorbance was read at 492 nm in ELISA reader (Bio-Rad, Benchmark plus).

#### 2.8. Competitive inhibition

Specific IgE inhibition assay for the identified peptides was performed to determine the allergenic potency and relative affinity of peptides toward cockroach hypersensitive patients' serum IgE. Briefly,  $100 \text{ ng}$  of Per a 10 was coated on ELISA plate and incubated overnight at  $4^\circ\text{C}$ . Cockroach hypersensitive pooled patients' sera ( $n=8$ ) was pre-incubated at  $4^\circ\text{C}$  overnight with different concentrations ( $0.01$ – $0.5 \mu\text{g}$ ) of B cell peptide B-P5, B-P6, B-P7 and Per a 10 separately and sera from healthy subjects were used as control (NHS). The plate was blocked with 1% BSA, then incubated with  $100 \mu\text{l}$  pre-incubated mixture of 1:10 v/v diluted cockroach hypersensitive pooled patient's sera (inhibited) overnight at  $4^\circ\text{C}$ . The plates were washed and incubated with anti-human IgE peroxidase (1:1000) v/v at  $37^\circ\text{C}$  for 3 h and processed further as in ELISA. The control was the serum pool without any inhibitor. Inhibition was presented as percentage decrease in absorbance of sample with inhibitor to that of sample without inhibitor and allergenic potency of peptide was defined by establishing the concentration of protein required for 50% ( $\text{IC}_{50}$ ) inhibition.

#### 2.9. Cytokine profile and lymphocyte proliferation by T cell peptides

Lymphocyte proliferation assay was performed as described elsewhere (Sharma et al., 2009). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-histopaque centrifugation (Sigma). Heparinized blood (5 ml) diluted 1:1 with PBS was layered onto an equal volume of histopaque (Sigma) and centrifuged at  $1000g$  for 30 min at  $25^\circ\text{C}$ . Upon centrifugation, PBMCs separate as a buffy layer at the interface of histopaque and plasma layers. PBMCs were recovered and washed twice with PBS and resuspended in complete RPMI-1640 media with 10% fetal calf serum (FCS) and cultured  $10^5$  cells/well in a 24 well plate. The cells were stimulated with Per a 10 ( $10 \mu\text{g}$ ), T cell peptides ( $10 \mu\text{g}$ ) and Phytohemagglutinin (PHA,  $5 \mu\text{g}$ ). PHA was used as positive control, whereas cells cultured with a control peptide (unrelated peptide  $10 \mu\text{g}$ ) served as negative control. The plates were cultured for 48 h at 5%  $\text{CO}_2$ . Culture supernatant was collected, centrifuged at  $1200g$  for 5 min and stored at  $-20^\circ\text{C}$  for further analysis. IL-4 and IL-5 levels were measured in culture supernatants as per the manufacturer's instruction (BD Pharmingen Opt EIA kits, BD Pharmingen, Germany). MTT assay on the cultured PBMCs was performed to assess lymphocyte proliferation. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT  $100 \mu\text{g}/\text{ml}$ , Sigma) was added to the PBMCs in the wells and incubated for 2 h at  $37^\circ\text{C}$ . Cells were lysed in acidic isopropanol and absorbance was read at 570 nm using ELISA reader (Bio-Rad, Benchmark plus).

### 3. Results

#### 3.1. Structure of Per a 10

Per a 10 sequence showed 61% similarity and 43% sequence identity with anionic trypsin from pacific chum salmon (*Oncorhynchus keta*). Four hundred structures simulating Per a 10 were generated using MODELLER 9v11. Model with the lowest DOPE score and GA341 score was selected for further analysis. The quality of model was assessed using the programs PROCHECK, ERRAT and Verify 3D in the SAVES server. The secondary structure prediction of Per a 10 sequence with PSIPRED server identified 4  $\alpha$

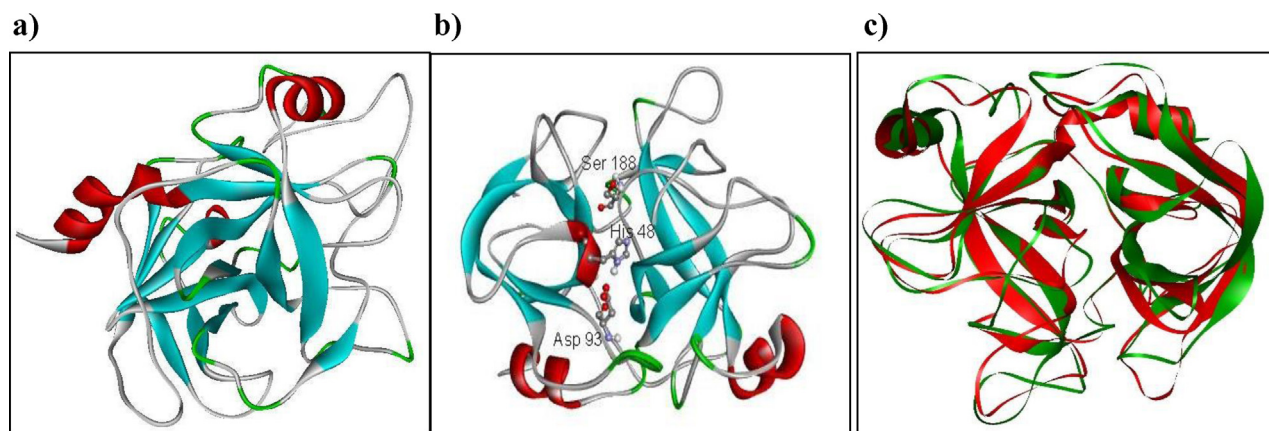


**Fig. 1.** Structure of Per a 10. (a) Homology modelled Per a 10 structure, (b) Per a 10 structure depicted with active site residues histidine, serine and aspartic acid in the catalytic cleft, (c) Target Per a 10 (in red) structure superimposed on template (in green) anionic trypsin from chum salmon to depict similarity in tertiary structure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

helices and 14  $\beta$  sheets. The  $\alpha$ -helices,  $\beta$ -pleated sheets and coils correlated well with the modelled structure of Per a 10 (Fig. 1a).

### 3.2. Quality assessment of Per a 10 model

#### 3.2.1. ERRAT

ERRAT is a quality assessment tool. Before optimisation, the region 1–24, 74–83, 121–126 displayed error values greater than 99% with a quality factor of 69.87% (S.Fig. 1a Supplementary material). In Per a 10 homology modelled structure, first 29 N-terminus amino acid residue region exceeded the error value in ERRAT and were therefore modelled as a loop by loop refinement function of MODELLER9v11. Hundred and fifty independent conformations were modelled and the best resulting model with the lowest DOPE score was selected and subjected for quality assessment. The resulting structure showed instability even after refining and therefore the sequences (first 22 residues) which lack homology with the template were deleted in a step wise fashion from the model by MODELLER 9v11 software. The overall quality factor of final refined structure came out to be 92.41% (S.Fig. 1b. Supplementary material).

#### 3.2.2. Procheck

Procheck is an online tool for stereochemical quality assessment of model. The main chain conformations of modelled protein were located in the acceptable regions of the Ramachandran plot. Majority of residues (96.7%) were in most favourable & allowed regions, 2.7% of the residues were in the generously allowed regions whereas 0.5% of the residues were in disallowed regions of the plot (S.Fig. 1b. Supplementary material). The resultant model was subjected to further optimization for energy minimization using the steepest descent algorithm implemented in the SYBYL 8.0 software.

#### 3.2.3. Verify 3D

Verify 3D assesses the compatibility of modelled 3-dimensional structure with its primary amino acid sequence. It showed 94% accuracy for the final model thus reinforcing the quality of predicted structure.

Per a 10 homology modelled structure was visualised by Discovery studio visualizer 3.0 software. The 3D structure was found to possess characteristic active site residues of serine proteases i.e. histidine, serine and aspartic acid residues in the catalytic cleft of the protein (Fig. 1b). The Per a 10 model was superimposed onto the template crystal structure of anionic trypsin protein from chum

salmon to check the folding similarity (Fig. 1c). The refined model was further used for epitope prediction.

### 3.3. B-cell epitope prediction

The B cell epitopes of Per a 10 were predicted by sequence and structure based methods. A consensus was taken among the commonly predicted IgE binding regions by various prediction tools used in the study. Seven antigenic regions were predicted in Per a 10. The predicted epitopes comprise of regions: B-P1: 27–41, B-P2: 86–97, B-P3: 132–145, B-P4: 154–164, B-P5: 175–186, B-P6: 198–211 and B-P7:231–240 [Table 1]. The most frequently occurring residues among the predicted epitopes were glycine (12.5%), followed by valine (10.5%). Hydrophobic content was analysed to identify regions with a higher probability of interaction with immunoglobulin. The B cell epitope B-P7 showed 27% hydrophobicity whereas B-P5 & B-P6 showed 8% hydrophobicity and therefore they are hydrophilic and have potentially high surface accessibility (Fig. 2b and 2d). Epitopes B-P1 (40%), B-P2 (23%), B-P3 (53%) and B-P4 (18%) showed comparatively high hydrophobicity [Table 1]. Spatial distribution of these epitopes on 3-D model of Per a 10 was done using Discovery studio visualizer 3.0, B-P1 and B-P2 were found to be comprised of loop and beta sheet structures, B-P3 and B-P4 epitopes lie in loop region and B-P5 comprises of alpha helix while B-P 6 and B-P7 occur in loop regions (Fig. 2b–d).

### 3.4. T cell epitope prediction

Seven publicly available tools on different datasets were employed for determination of HLA-DR, DP, and DQ binders. Three T cell epitope regions T-P8: 4–14, T-P9: 214–219 and T-P10: 240–250 [Table 1] were identified as promiscuous binders of MHC II by taking consensus from the prediction data. The predicted T cell epitopes mostly composed of alanine, lysine and isoleucine. The epitopes are localized both on the surface and interior of the protein molecule. The T cell epitopes comprised of  $\alpha$ -helix (T-P10),  $\beta$  sheets (T-P9) and loop (T-P8) (Fig. 2c). T cell epitope T-P10 overlaps with one amino acid residue of B cell epitope B-P7 (Fig. 2d) while the other two epitopes T-P8 and T-P9 do not overlap with any of the predicted B cell epitopes. Among the three T cell epitopes, T-P8 and T-P10 predicted by MHCpred, SVRMHC and SMM-Align displayed low IC<sub>50</sub> binding value indicating high affinity to the HLA-DR alleles DRB\*0101, DRB\*0401, DRB\*0701, DRB\*1501 by quantitative prediction methods. RANKPEP and SYFPEITHI also showed high percentile value for T cell epitopes T-P8 and T-P10 indicating high affinity to the alleles DRB\*0101, DRB\*0401, DRB\*0701 and

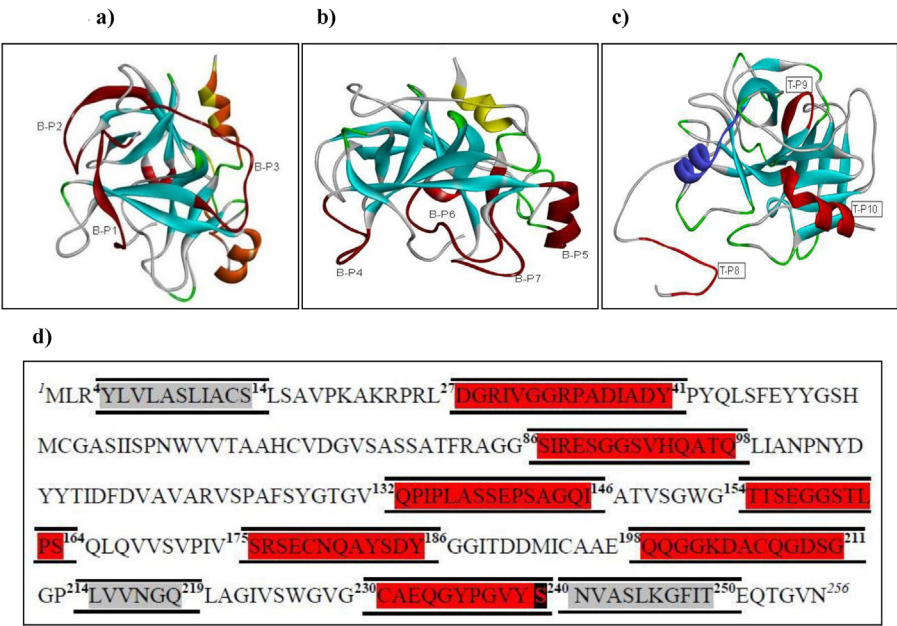


**Fig. 2.** Distribution of predicted B and T cell epitopes. Per a 10 structure depicting (a) and (b) predicted B cell epitopes, (c) predicted T cell epitopes and (d) Linear amino acid sequence of Per a 10 depicting predicted epitopes. Highlighted Red: B cell epitopes, Grey: T cell epitopes.

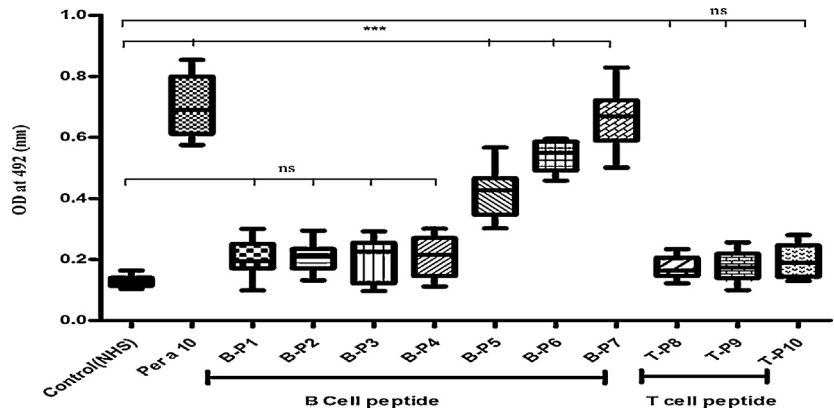


**Fig. 3.** IgE binding studies. IgE binding of B and T cell peptides was analysed with individual cockroach hypersensitive patients' sera. Per a 10 or peptides were coated on ELISA plate and incubated with ten individual cockroach hypersensitive patients' sera. The IgE affinity was detected by HRP labelled anti-human IgE. The data is presented as box and whiskers plot showing variation of IgE values from median. \*\*\*p < 0.0001, ns: not significant.

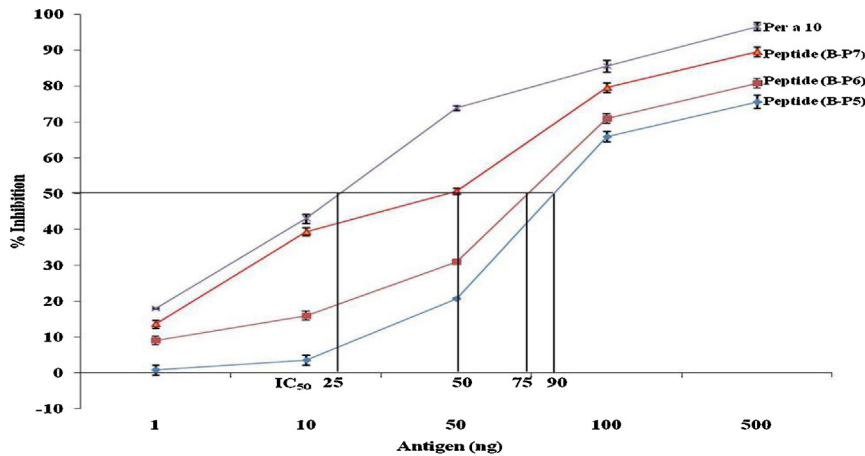


**Fig. 4.** ELISA inhibition assay. Competitive inhibition demonstrated by Per a 10, B-P5, B-P6 and B-P7 to surface bound Per a 10 and expressed as percentage inhibition. Per a 10 protein and B cell peptides were pre-incubated with pooled cockroach hypersensitive patients' sera (n = 8) in increasing concentrations (ranging from 1 to 500 ng) and added onto microtiter plates coated with 100 ng of Per a 10 protein in solid phase. The IgE affinity was detected by HRP labelled anti-human IgE .

**Table 1**

IgE binding studies on predicted epitopes using pooled cockroach positive patients' sera. IgE binding of peptides were tested by ELISA using pooled cockroach hypersensitive patients' sera (n = 6). The control serum samples were drawn from healthy individuals. The specific IgE values represented here are mean values of triplicates.

| Peptide         | Sequence        | Size (mer) | Position in Per a 10 protein | Hydrophilicity Index | Hydrophobicity Index | IgE binding (OD at 492) |
|-----------------|-----------------|------------|------------------------------|----------------------|----------------------|-------------------------|
| B-P1            | DGRIVGGRPADIDY  | 15         | 27–41                        | 33%                  | 40%                  | 0.324 ± 0.021           |
| B-P2            | SIRESGGSVQATQ   | 13         | 86–98                        | 15%                  | 23%                  | 0.251 ± 0.035           |
| B-P3            | QPIPLASSEPSAGQI | 15         | 132–146                      | 7%                   | 53%                  | 0.292 ± 0.023           |
| B-P4            | TTSEGGSTLPS     | 11         | 154–164                      | 9%                   | 18%                  | 0.315 ± 0.015           |
| B-P5            | SRSECNQAYS DY   | 12         | 175–186                      | 25%                  | 8%                   | 0.491 ± 0.026           |
| B-P6            | QQGGKDACQGD SG  | 13         | 198–211                      | 23%                  | 8%                   | 0.505 ± 0.017           |
| B-P7            | CAEQGYPGVYS     | 11         | 230–240                      | 9%                   | 27%                  | 0.709 ± 0.031           |
| T-P8            | YLVLASLIACS     | 11         | 4–14                         | 0%                   | 64%                  | 0.209 ± 0.027           |
| T-P9            | LVVNGQ          | 6          | 214–219                      | 0%                   | 50%                  | 0.192 ± 0.015           |
| T-P10           | SNVASLKG FIT    | 11         | 240–250                      | 9%                   | 45%                  | 0.208 ± 0.035           |
| Per a 10        | –               | 256        | –                            | 33%                  | 43%                  | 1.093 ± 0.041           |
| C E             | –               | –          | –                            | –                    | –                    | 1.886 ± 0.025           |
| Control Peptide | YVGNRKDSSE AIEF | 14         | –                            | 21%                  | 35%                  | 0.128 ± 0.031           |

**Table 2**

HLA-DR allele and IC<sub>50</sub> binding score of predicted T cell epitopes. Predicted T cell epitopes T-P8 and T-P10 showed less IC<sub>50</sub> binding by MHCpred, SMM-align and SVRMHC, and high percentile values by RANKPEP and SYFPEITHI prediction tools indicating high affinity binding to the HLA-DR alleles DRB\*0101 and DRB\*0401.

| Peptide | sequence     | Allele   | MHCpred IC <sub>50</sub> binding Score | SMM-align IC <sub>50</sub> nM binding Score | SVRMHC pIC50 binding score | RANKPEP Optimal score (%) | SYFPEITHI Binding Score (%) |
|---------|--------------|----------|----------------------------------------|---------------------------------------------|----------------------------|---------------------------|-----------------------------|
| T-P8    | YLVLASLIACS  | DRB*0101 | 8.575                                  | 3                                           | 7.990                      | 41.95                     | 32                          |
|         |              | DRB*0401 | 6.508                                  | 91                                          | 7.167                      | 20.45                     | 28                          |
|         |              | DRB*0405 |                                        | 122                                         | 6.768                      |                           |                             |
|         |              | DRB*0501 |                                        |                                             | 5.850                      |                           |                             |
|         |              | DRB*0701 | 7.093                                  | 64                                          |                            | 26.40                     | 22                          |
| T-P10   | SNVASLKG FIT | DRB*1501 |                                        | 93                                          | 6.448                      |                           | 20                          |
|         |              | DRB*0101 | 7.325                                  | 31                                          | 7.118                      | 19.78                     | 30                          |
|         |              | DRB*0401 | 6.385                                  | 230                                         | 6.726                      | 25.41                     | 28                          |
|         |              | DRB*0405 |                                        | 163                                         | 6.430                      | 40.73                     |                             |
|         |              | DRB*0501 |                                        |                                             | 6.119                      |                           |                             |
|         |              | DRB*0701 | 6.512                                  | 94                                          |                            | 32.04                     | 22                          |
|         |              | DRB*1501 |                                        | 188                                         | 6.463                      |                           | 24                          |

DRB\*1501 [Table 2]. Peptide T-P9 showed high IC<sub>50</sub> and less percentile binding values by the above mentioned alleles (data not shown).

### 3.5. Enzyme linked immunosorbent assay

#### 3.5.1. ELISA with pooled cockroach hypersensitive sera

Synthetic peptides representing the epitopes were tested for IgE binding by ELISA using pooled (n = 6) sera. CE showed highest IgE binding followed by Per a 10. Predicted B cell epitopes also showed IgE binding with pooled cockroach hypersensitive patients' sera however the levels were lower in comparison with CE and Per a 10. T cell peptides showed almost negligible IgE binding [Table 1].

#### 3.5.2. ELISA with individual cockroach hypersensitive sera

The specific IgE binding of B and T cell peptides and Per a 10 was assessed by ELISA using individual cockroach hypersensitive patients' sera (n = 10). ELISA positive was considered as OD ≥ 3 times of the control NHS (Normal Human Serum). Among seven B cell peptides, three peptides i.e. B-P5, B-P6 and B-P7 reacted with all the ten cockroach hypersensitive patients' sera and showed significantly high IgE binding ( $p \leq 0.0001$ ) compared to other B cell peptides B-P1 to B-P4 which demonstrated no statistical difference in IgE binding compared to NHS. Individual cockroach hypersensitive patients' sera showed high IgE value against Per a 10 compared to B cell peptides. T cell peptides as expected did not show IgE binding (Fig. 3).

### 3.6. Competitive inhibition

ELISA inhibition assay was performed to check the allergenic potency and specificity in IgE binding of B cell peptides: B-P5, B-P6 and B-P7 along with whole allergen Per a 10. The dose dependent inhibition of IgE binding to the solid phase coated Per a 10 was observed. Pooled cockroach hypersensitive patients' sera (n = 8) were incubated with different concentration of (0.01–0.5 µg) of B cell peptides: B-P5, B-P6, B-P7 and Per a 10. Per a 10 (self) showed maximum of 96% inhibition while B-P7 showed 89% inhibition and B-P5 & B-P6 showed 75% and 80% inhibition respectively. For inhibition of solid phase bound Per a 10 protein, 50 ng of B-P7 was required compared to 75 ng and 90 ng of B-P5 & B-P6 peptides respectively for 50% inhibition in IgE binding. IC<sub>50</sub> inhibition for (ELISA self-inhibition) Per a 10 was attained at 26 ng of protein (Fig. 4).

### 3.7. Lymphocyte proliferation assay on PBMCs and cytokine profiling

#### 3.7.1. Lymphocyte proliferation by T cell epitopes

PBMC from cockroach hypersensitive patients' (n = 15) showed a significant lymphocyte proliferation on stimulation with T cell epitopes or Per a 10 or CE. The predicted T cell peptides: T-P8, T-P10 and Per a 10 showed significantly higher PBMC proliferation ( $p \leq 0.001$ ) compared to negative control i.e. control peptide, whereas T-P9 showed no significant difference to control peptide. T cell peptides: T-P8, T-P9 and T-P10 induced lower PBMC prolifer-

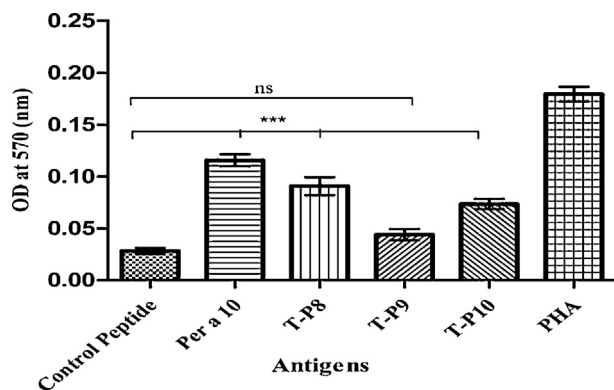


**Fig. 5.** Lymphocyte proliferation assay. PBMCs isolated from cockroach hypersensitive patients' ( $n = 15$ ) were stimulated with  $10 \mu\text{g}$  of native Per a 10 or T cell peptides: T-P8, T-P9 or T-P10 and cultured for 48 h in RPMI with 10% fetal calf serum. Phytohemagglutinin (PHA,  $5 \mu\text{g}$ ) was used as positive control for stimulation, while cells stimulated with control peptide served as negative control. The proliferation was measured by MTT assay. \*\*\* $p \leq 0.0001$ , ns: not significant.

eration as compared to Per a 10 ( $p \leq 0.0001$ ) and positive control PHA (Fig. 5).

### 3.7.2. Cytokine profile

IL-4 and IL-5 cytokines were detected in the supernatant of all the cockroach hypersensitive patients' PBMCs ( $n = 15$ ) on stimulation with CE or Per a 10 or T cell epitopes.

The induction in IL-4 levels by T cell peptides were lower than Per a 10 ( $p \leq 0.0001$ ). A significant increase in IL-4 levels was observed upon stimulation with T-P8, T-P9, T-P10 and Per a 10 as compared to control peptide ( $p \leq 0.001$ ). (Fig. 6a).

CE showed the highest levels of IL-4 in the culture supernatant (data not shown).

Significant levels of IL-5 were observed upon stimulation with T cell peptides: T-P8, T-P9, T-P10 and Per a 10 compared to control peptide ( $p \leq 0.0001$ ). The induction in IL-5 levels by T cell peptides was lower than Per a 10 (Fig. 6b). CE induced highest levels of IL-5 (data not shown). Among the three T cell peptides, T-P8 and T-P10 induced higher IL-4 and IL-5 levels in comparison to T-P9 in PBMCs of cockroach hypersensitive patients'.

## 4. Discussion

Three dimensional structure is a pre-requisite in understanding the structure function relationship of allergen antibody responses and epitope identification (Pomés, 2010). Previously, the structure of Bet v 1 allergen was determined by X ray crystallography and NMR techniques (Gajhede et al., 1996). The structure determination further aided in identification of immunodominant epitope of Bet v 1 and its subsequent characterisation by site directed mutagenesis (Spangfort et al., 2003). Methods such as generation of overlapping peptides, site directed mutagenesis, T cell lines and clones, screening of libraries of overlapping peptides have been utilised for determination of B and T cell epitopes (Frazier et al., 2014). Although accurate, these methods are laborious and time consuming. With an expansion in knowledge and data about structures and epitopes of known allergens, computational algorithms are being developed. These computational tools are fast, precise and cost effective means of structure determination and mapping of antigenic determinations (Frazier et al., 2014; Yang and Yu, 2009). Previously, Cur l 3 was homology modelled and its B and T cell epitopes were predicted by *in-silico* tools and validated (Sharma et al., 2009).

In the present study, Per a 10, a major serine protease allergen, structure was modelled based on the template anionic trypsin from pacific chum salmon (PDB ID: 1mbq). The model showed permissible bond angles and Van der Waals radii. The stereochemical evaluation by SAVES server demonstrated it to be a good model with an overall quality factor of 92.41% in ERRAT. Earlier, the structure of ADH from *Curvularia lunata* was *in-silico* predicted and it was shown that computational tools employed for three dimensional structure determination provide good approximation and can be utilised for epitope prediction (Nair et al., 2011). Recently, B cell epitopes of peanut allergens namely Ara h 1, 2, 3, 5, 6, 7, 8, 9, 10 and 11 were identified by *in-silico* methods. The *in-silico* predicted epitopes correlated with the experimentally identified epitopes of Ara h 1, 2 and 3 (Mishra et al., 2016). Similarly, in another study B and T cell epitopes of Der f 25, a house dust mite allergen, have been identified by computational methods (Li et al., 2014).

For B and T cell epitope prediction, consensus method was employed as it combines the strengths of different prediction tools to enhance the specificity and sensitivity of prediction (Yang and Yu, 2009). B cell epitopes were predicted by sequence and structure based tools. The intrinsic biochemical properties of the epitopes

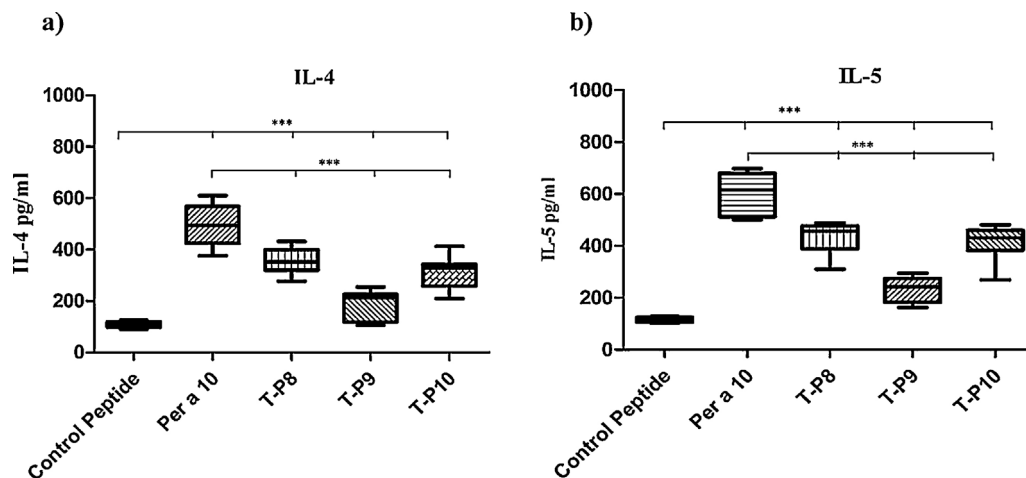


**Fig. 6.** Th2 Cytokine profile. (a) IL-4 and (b) IL-5 cytokines secreted in supernatant of cockroach hypersensitive patients' PBMCs ( $n = 15$ ) cultured in the presence of  $10 \mu\text{g}$  of Per a 10 or peptides were measured by ELISA. Cells stimulated with control peptide served as negative control. The experiments were performed in triplicates and average was taken. The box graph represents here deviation from median value. Control samples were drawn from healthy volunteers. \*\*\* $p \leq 0.0001$  as compared to control and Per a 10.

such as hydrophobicity, hydrophilicity and overall charge affects IgE binding potential (Nair et al., 2011). Studies with pooled cockroach hypersensitive patients' sera demonstrated that peptides with correspondingly high hydrophilicity and low hydrophobicity (B-P5 and B-P6) showed higher IgE binding than unrelated control peptide [Table 1]. The highest IgE binding among the predicted peptides was shown by B-P7 despite having considerably high hydrophobicity and low hydrophilicity. Its IgE binding potential may be attributed to presence of tyrosine residues which are known to form protein interface hubs (Brinda et al., 2005). A study has shown that amino acids like Ala, Ser, Asn, Gly and Lys possess high propensity of occurrence in B cell epitopes (Oezguen et al., 2008). According to another study, Cys and Glu predominates in IgE binding epitopes (Gupta et al., 2013). In case of Per a 10, specific enrichment of Gly, Pro, Cys and Glu residues occurred on high IgE binding epitope peptides B-P5, B-P6 and B-P7. These peptides showed IgE binding with all the ten patients' sera included in the study therefore, substantiating the prediction efficiency of *in-silico* tools. ELISA inhibition assay was performed with high IgE binding B cell peptides (B-P5, B-P6 and B-P7) to assess their potency. In comparison to Per a 10 protein which requires 26 ng for self-inhibition and showed the highest percentage inhibition of 96%, peptide B-P7 (89%) showed maximum inhibition amongst predicted B cell epitopes followed by B-P6 (80%) and B-P5 (75%) with corresponding IC<sub>50</sub> values of 50 ng, 90 ng and 75 ng respectively.

Allergen-specific CD4<sup>+</sup> T cells play a crucial role in inducing allergic immune responses and are targets for immunotherapeutic intervention in allergic diseases (Tanabe, 2007). Immunotherapy with short peptides (T cell epitopes) is more effective than crude allergen mixtures or purified protein (Larché, 2007). The short synthetic peptides representing major T cell epitopes have shown promising results in preclinical studies (Oldfield et al., 2002; Müller et al., 1998). Administration of high dose of allergen specific T cell epitope peptides downregulates adverse Th2 polarized response to allergen via induction of anergy and upregulation of T<sub>reg</sub> responses with IL-10 production (Prickett et al., 2015). HLA class II molecules are highly heterogeneous and polymorphic. These are encoded by four different loci with hundreds of allelic variants in general population. Computational approach for T cell epitope prediction relies on identification of promiscuous epitopes capable of cross binding multiple HLA class II molecules (Frazier et al., 2014). Certain algorithms have been developed to predict T-cell epitopes with improved prediction accuracy (Kim et al., 2012). Earlier, HLA-DR ligands of Lol p 5a allergen from rye grass were identified by TEPI-TOPE prediction method (de Lalla et al., 1999). Recently, T cell epitopes of cockroach allergens Per a 6 and Bla g 6 were predicted by *in-silico* tools (Chen et al., 2014).

In case of Per a 10, three T cell epitopes were predicted by binding score and inhibitory concentration dependent tools. These epitope peptides showed high hydrophobicity and negligible IgE binding in ELISA [Table 1]. Out of the three T cell peptides, T-P8 and T-P10 were identified as potential HLA II ligands for DRB\*0101 and DRB\*0401 as predicted by MHCpred, SVRMHC, SMM-Align, RANKPEP and SYFPEITHI online tools [Table 2].

*In vitro* expansion of CD4<sup>+</sup> T cells and analysis of culture supernatant is an effective strategy to assess the T cell effector functions of predicted epitope peptides (Van Hemelen et al., 2015). The promiscuous HLA binders identified by the above mentioned tools (T-P8 and T-P10) induced significant lymphocyte proliferation in PBMCs of cockroach hypersensitive patients' along with high levels of IL-4 and IL-5 cytokine in the culture supernatant. The allergen dependent stimulation of PBMCs was specific for the predicted epitopes as unrelated control peptide showed negligible proliferation in patients' PBMCs. Previously, T cell epitopes of the *Blattella germanica* allergens, Bla g 1, 2, 4, 5, 6, and 7 have been predicted by *in-silico* tools in conjunction with PBMC proliferation studies

(Oseroff et al., 2012). A recent study has identified T cell epitopes of food allergen Ara h 1 by *in-silico* methods. The predicted epitopes were validated by PBMC proliferation assay and Th2 cytokine production, and the results of *in-silico* and *in vitro* experiments showed high reproducibility (Ramesh et al., 2016).

Of the seven predicted B cell epitopes, three (B-P5, B-P6 and B-P7) peptides demonstrated considerable IgE binding. Comparing the prediction efficacy of different servers, in case of B cell epitopes, structure based epitope prediction softwares DiscoTope and CEP have predicted all the three high binders (B-P5, B-P6 and B-P7) correctly. Although sequence based, BepiPred 1.0b and ElliPro servers have also predicted the high IgE binders, while other sequence based servers BCPREDS, Antigenic, ABCpred and DNASTAR have predicted the B cell epitopes partially. For T cell epitopes, correlation among IC<sub>50</sub> based tools and binding score dependent methods was observed for T-P8 and T-P10. T-P9 although predicted by consensus from the mentioned tools, failed to elicit T cell responses in *in vitro* studies. Despite being a short peptide T-P9 was included in the study as it was predicted by almost all the prediction servers. The current observations underscore the need of appropriate experimental validation of *in-silico* predicted data.

In summary, Per a 10 protein was homology modelled and its three B cell epitopes and two T cell epitopes were identified by specific IgE binding and PBMC proliferation assays. The *in-silico* approach in conjunction with *in vitro* assessment provides an efficient strategy for epitope prediction and can be further utilised for diagnosis and therapeutics.

## Conflict of interest

The authors declare no conflict of Interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.molimm.2016.10.007>.

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