

Bioinformatic and immunological investigation of Per a 5 (delta class GST) allergen from *Periplaneta americana*

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ABSTRACT

Introduction: GSTs are multifunctional enzymes involved in cellular detoxification and present as potent allergens in several sources. Present study investigates allergenic relevance of GST from *P. americana* and determine its cross reactive potential with other indoor allergen sources.

Methods: Computational analysis with FASTA and ConSurf webserver was performed to determine potentially cross reactive allergens. Further, Per a 5 gene was cloned in pET 22b+ vector and expressed in *E. coli* BL21 cells and the rPer a 5 protein was purified using Ni-NTA affinity chromatography. Enzymatic activity of rPer a 5 was assessed using CDNB and cumene hydroperoxide. ELISA and immunoblot were performed using cockroach hypersensitive patient's sera. Functional activity of rPer a 5 was evaluated by basophil activation test. Inhibition studies were carried out with *D. pteronyssinus*, *A. alternata* and *C. lunata* extracts.

Results: Per a 5 demonstrates highest sequence similarity with delta class GST of *Blattella germanica* (94.9%). It also exhibits significant sequence similarity (50–58%) with mite, fungal and helminth allergenic GSTs. ConSurf analysis reveals high degree of evolutionary similarity in N terminal region of Per a 5, especially at GST dimerization interface. The purified rPer a 5 protein resolved at 27 kDa on SDS-PAGE. The rPer a 5 protein exhibits GST activity and possess upto 65% immunoreactivity with cockroach hypersensitive patient's sera in ELISA and immunoblot. It upregulates expression of CD203c on basophils signifying its biological ability to activate effector cells. rPer a 5 significantly inhibits corresponding GSTs in *P. americana*, *D. pteronyssinus*, *A. alternata* and *C. lunata* with EC₅₀ values of 15.5 ng, 38.38 ng, 41.4 ng and 61.66 ng, respectively.

Conclusion: Recombinant delta class GST of *P. americana* is a clinically relevant allergen showing upto 65% immunoreactivity with hypersensitive patient's sera. Per a 5 GST allergen showed phylogenetic similarity with dust mite, fungal and birch allergens thereby demonstrating allergen cross reactivity.

1. Introduction

Cockroaches are significant source of allergenic proteins and are strongly associated with severity of allergic asthma in children and young adults (Pomes and Arruda, 2014; Pomés et al., 2019). *Periplaneta americana* demonstrates widespread distribution and is the principal allergic sensitizer in tropical and sub-tropical regions (Sookrung and Chaicumpa, 2010). It consists of twenty two IgE binding proteins of which nine allergens have been identified (Sudha et al., 2008; Pomes and Arruda, 2014). Among these myriad of allergenic proteins, Glutathione-S-transferases (GSTs) represent a group of protective enzymes with pronounced allergenic potential. Studies have identified

clinically relevant GST allergens from a variety of sources including dust mite, pollen, fungi and german cockroach and helminths such as *Wuchereria bancrofti*, *Ascaris lumbricoides* (O'Neill et al., 1995; Arruda et al., 1997; Deifl et al., 2014; Shankar et al., 2006; Santiago et al., 2012; Acevedo et al., 2013).

GSTs are ubiquitously expressed proteins of approximately 25–30 kDa size and are known to exist as homo/hetero dimers. GSTs perform phase II detoxification of numerous xenobiotic compounds and endogenous products of oxidative stress. Considering their cytoprotective nature, insect GSTs belonging to delta and epsilon classes of GSTs form the fundamental defense systems against insecticides (Meng et al., 2019; Lumjuan et al., 2005). In addition, GSTs are highly induced in

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insecticide resistant strains of insects implying their functional role in encountering xenobiotic stress and improving survival (Enayati et al., 2005).

Allergens displaying ubiquitous distribution tend to present cross reactive potential. GSTs from several fungal sources are known to be cross reactive (Shankar et al., 2005). A recent study identified cockroach as a principal cross reactive allergen in shrimp sensitized individuals, however the associated cross reactivity was not due to tropomyosin, thereby indicating towards presence of other potentially cross reactive allergens in cockroach. An earlier study by Huang et al. have shown Der p 8 allergen to be cross reactive with cockroach GSTs (Huang et al., 2006). Tropical and sub-tropical environment provides an eclectic mixture of several co-sensitizing allergen sources. Assessment of cross-reacting and co-sensitizing allergenic proteins is necessary for accurate molecular diagnosis. We aimed to employ a combination of computational tools and recombinant DNA technology to characterize Per a 5 protein and determine its allergenic relevance and cross reactive potential. The purified protein may possess potential for component resolved diagnostics and therapeutic strategies (Pomés et al., 2019).

2. Methods

2.1. Computational analysis of allergenicity and conservation analysis

2.1.1. Allergenicity assessment of Per a 5

The amino acid sequence of Per a 5 was retrieved from UniProt (Q1M0Y4) and subjected to FASTA alignment in AllergenOnline and SDAP databases (<http://www.allergenonline.org/>) (Ivanciuc et al., 2003), (<https://fermi.utmb.edu/>). Multiple sequence alignment (MSA) was performed using MUSCLE tool (<https://www.ebi.ac.uk/Tools/msa/muscle/>) (Madeira et al., 2019).

2.1.2. Sequence retrieval and phylogenetic analysis

GST protein sequences were retrieved from UniProt database and aligned using MUSCLE tool. Phylogenetic analysis was performed using Neighbor-joining method in MEGA X tool (Kumar et al., 2018). Multiple sequence alignment with GST proteins was submitted to ConSurf web-server (Ashkenazy et al., 2016; Celniker et al., 2013). Conservation analysis of amino acid residues was performed by Rate4Site algorithm in ConSurf webserver (Pupko et al., 2002). The evolutionary rates were grouped as conservation grades ranging from 9 to 1 (9 being most conserved and 1 being most variable) and mapped onto Per a 5 structure (generated using ConSeq mode in ConSurf) using Chimera 1.13.1 software (Pettersen et al., 2004). To gain an insight into evolutionary conservation of residues involved in dimerization, homodimer of Per a 5 was constructed by Oligomerization function of SWISS-MODEL using template structure of *Drosophila* GST dmGSTD1 (Uniprot ID 3mak.1) exhibiting 62.5 % sequence identity with Per a 5 (Waterhouse et al., 2018; Bertoni et al., 2017).

2.2. Cloning of Per a 5 gene

Total RNA was isolated from freeze dried cockroach powder using EZ-RNA isolation kit (Biological Industries Sartorius) and converted to cDNA by RevertAid (H-) cDNA synthesis kit (ThermoFisher). Per a 5 gene (Genbank accession number: AY792949.1) was PCR amplified using gene specific primers. The PCR product and vector backbone were cleaved by Eco R I and Xho I, gel eluted by QIAquick gel extraction kit (Qiagen, Germany) and ligated using T4 DNA Ligase (ThermoFisher) at 16 °C overnight. Ligation mixture was transformed in *E.coli* DH5α cells and positive colonies were grown overnight at 37 °C. Restriction digestion of positive colony showed the presence of 651 bp insert. The clone was also confirmed by gene sequencing. Forward primer: 5'GTATTGGAATTCGATGACCATCGACTTCTAC 3'. Reverse primer: 5'ACTGAACGAGCTTCTTGGCGAGGTTATC 3'

2.3. Expression, Purification and characterization of rPer a 5 allergen

2.3.1. Purification of rPer a 5

Recombinant Per a 5 (rPer a 5) was expressed in *E.coli* BL21 λ DE 3 cells. Briefly, the clone containing Per a 5 was grown until OD_{600nm} reached 0.5 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma Aldrich) for 4 h followed by harvesting. The cell pellets obtained were dissolved in binding buffer (50 mM Tris, 300 mM NaCl), sonicated and centrifuged. Meanwhile, Ni-NTA agarose (Qiagen, Germany) resin was equilibrated with binding buffer and incubated with supernatant for 2 h at 4 °C. The column was washed with wash buffer (binding buffer with 25 mM imidazole) and eluted with elution buffer (binding buffer containing 250 mM imidazole). Protein elutes were concentrated and dialyzed with Amicon concentrator (Millipore) and re-purified with Ni-NTA resin. The re-purified elutes were concentrated and resolved as a single band at approximately 27 kDa on 12% SDS-PAGE.

2.3.2. Biochemical characterization

The rPer a 5 protein was probed with anti-GST and anti-Histidine antibodies (Sigma Aldrich). Briefly, the rPer a 5 protein was resolved on SDS PAGE and transferred overnight onto PVDF membrane. The membrane was blocked by 3% BSA in TBS for 2 h at room temperature and washed with TBST and incubated with anti-Histidine peroxidase antibody (at 1:10,000 dilution) or anti-GST peroxidase antibody (at 1:10,000) in 1% BSA in TBS for 1 h at room temperature. The blots were washed with TBST and then developed using Supersignal West Femto Substrate (ThermoFisher).

2.4. Specific IgE ELISA

ELISA was performed with cockroach extract (CE, prepared as previously described (Govindaraj et al., 2013)) and rPer a 5. Briefly, 100 ng antigen was coated per 100 μL of bicarbonate buffer per well in microtiter plates and kept at 4 °C overnight. The plates were washed with PBS and blocked with 3% BSA in PBS for 2 h at room temperature. The plates were then washed with PBST and individual serum samples (n=15 of cockroach hypersensitive patient's sera and n=2 for normal human sera, diluted 1:10 in 1% BSA in PBS) were added into the wells in duplicates and kept for overnight incubation at room temperature. The plates were again washed with PBST and incubated with anti-human IgE peroxidase (Sigma Aldrich) antibody (diluted 1:1000 in 1% BSA in PBS) for 30 min at room temperature. Plates were washed with PBST and then developed with TMB (Sigma Aldrich). Reaction was stopped by addition of 2N H₂SO₄ and absorbance was measured at 450 nm on Thermo Multiskan GO spectrophotometer.

2.5. Immunoblot

The protein rPer a 5 was resolved on SDS PAGE and transferred overnight onto nitrocellulose membrane. The membrane was blocked by 3% BSA in PBS for 2 h at room temperature and washed with PBST. The membrane was incubated with cockroach hypersensitive patient sera (n=15) and normal human sera (n=2), 1:20 diluted in 1% BSA in PBS and incubated overnight at 4 °C. The membrane was washed with PBST and incubated with anti-human IgE peroxidase (Sigma Aldrich) antibody (diluted 1:1000 in 1% BSA in PBS) for 30 min at room temperature. The blots were washed with PBST and then developed using Supersignal West Femto Substrate (ThermoFisher).

2.6. GST activity assay

GST activity assay for Per a 5 was performed with both CDNB and Cumene hydroperoxide as substrates. Bovine serum albumin was used as a negative control for both activity assays. For CDNB based GST assay, equine liver GST was used as a positive control. The rPer a 5 protein was

reconstituted in sample buffer (100 mM potassium phosphate buffer, pH 6.5 containing 0.1% Triton x-100 and 1 mM GSH). Briefly, in a 96 well plate, proteins were diluted with assay buffer (100 mM Potassium phosphate buffer, pH 6.5 containing 0.1 % Triton x-100) followed by addition of 200 mM glutathione to the reaction mixture. Reaction was initiated by addition of 100 mM CDNB and absorbance was read every minute at 340 nm for 10 minutes on Thermo Multiskan GO spectrophotometer. GST activity was calculated by using the following formula:

$$\text{Rate of reaction } \Delta A_{340}/\text{min} = [A_{340}(\text{Time } 2) - A_{340}(\text{Time } 1)] / [\text{Time } 2 (\text{min}) - \text{Time } 1 (\text{min})]$$

$$\text{GST activity} = [\Delta A_{340\text{nm per min}} \times 0.2\text{ml}] \div [0.0053\mu\text{M}^{-1} \times 0.02\text{ml}] \times \text{sample dilution}$$

Activity of rPer a 5 with cumene hydroperoxide (a substrate for Glutathione peroxidase) was performed using Glutathione Peroxidase Assay kit (Cayman Chemical) following manufacturer's instructions. Bovine erythrocyte glutathione peroxidase was used as positive control for assay. Peroxidase activity was calculated using the formula: $[(\Delta A_{340}/\text{min})/0.00373] \times [0.19/0.02]$

2.7. Basophil activation test

A passive sensitization protocol was used for basophil activation test (Santos et al., 2015). Briefly, PBMCs were isolated from healthy volunteers using Histopaque (Sigma Aldrich). PBMCs were resuspended in lactic acid solution (13.4 mM Lactic acid, 140 mM NaCl and 5 mM KCl) for 5 minutes at 4 °C to strip receptor bound IgE. The reaction was stopped by addition of RPMI media containing 0.25% Human serum albumin (HSA, Sigma Aldrich) and washed with the same solution. The cell pellet was resuspended in HSA supplemented RPMI media and incubated with pooled serum samples of cockroach hypersensitive patients for 1 h at 37 °C. After washing, the re-sensitized PBMCs were stimulated with CE (2 ng/μl) and Per a 5 (2 ng/μl) for 30 minutes at 37 °C. Anti-human IgE (Sigma Aldrich) at dilution of 1:200 and FMLP (formylmethionyl-leucyl-phenylalanine) (Sigma Aldrich) at concentration of 1 μM were used as positive controls while the unstimulated samples served as negative control. The cells were washed and stained with PE anti-human CCR3 and APC-anti human CD203c (BD Pharmingen). Basophils were selected as CCR3⁺ cells. Basophil activation is expressed as increase in CD203c levels in CCR3⁺ basophils.

2.8. Inhibition studies

ELISA inhibition was performed with extracts of *Periplaneta americana*, *Dermatophagoides pteronyssinus*, *Alternaria alternata* and *Curvularia lunata*. *D. pteronyssinus* extract was prepared in 20 mM Ammonium bicarbonate buffer, pH 8.0 containing 2 mM EDTA and 1 mM PMSF for four hours and was centrifuged, filtered, dialysed and lyophilized. Fungal cultures and extracts were prepared as described previously (Shankar et al., 2005). Qualitative and quantitative analysis was done by SDS-PAGE and protein estimation (by BCA) respectively. Briefly, 250 ng of rPer a 5 protein was coated per 100 μL of bicarbonate buffer per well in microtiter plates and kept at 4 °C overnight. Sera samples (n=6) with high IgE titres were pooled and incubated with increasing amounts of allergen extracts (0.1 ng–250 ng per well) and kept at 4 °C overnight. The plates were washed with PBS and blocked with 3% BSA in PBS for 2 h at 37 °C. The plates were then washed with PBST and inhibited serum samples were added into the wells in duplicates and kept for 2 h at 37 °C. The plates were again washed with PBST and incubated with anti-human IgE peroxidase (Sigma Aldrich) antibody (diluted 1:1000 in 1% BSA in PBS) for 30 minutes at room temperature. Plates were washed and developed as described in section 2.4.

2.9. Statistical analysis

Statistical analysis of the results was done using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Allergenicity and molecular conservation analysis of Per a 5

3.1.1. Allergenicity assessment of Per a 5

FASTA search in AllergenOnline database revealed highest sequence similarity of Per a 5 with delta class GST of *Blattella germanica* (94.9%). It demonstrated sequence similarity in range of 50–58% with mite GSTs Der f 8, Blo t 8, itch mite GST, fungal GST (Alt a 13) and sigma class GST from *Ascaris suum*. Among plants, Per a 5 showed 52% similarity with GST from *Triticum aestivum* (wheat) and *Betula pendula* (silver birch) (Supplementary Table 1). Multiple sequence alignment of Per a 5 with allergenic proteins (demonstrating highest sequence similarity in Allergen Online and SDAP databases) showed presence of stretches of conserved sequences interspersed with sequences of low conservation (Fig. 1a). The N terminal of protein is comparatively more conserved than C terminal which may be attributed to presence of GSH binding site and substrate binding site in N- and C- terminal respectively. The apparent variation in C terminal region may be ascribed to the ability of GSTs to bind a diverse range of substrates. The conserved stretch of amino acids in N terminal of Per a 5 consists of residues Pro₅₅, Glu₆₆, Ser₆₇ and Leu₇₄ constituting dimer interface 1. Among these, the Proline at position 55 is observed in all the insect GST sequences analysed. Other studies also indicate its important role in structural formation in glutathione binding site (Oakley et al., 2009). Another conserved stretch comprising of substrate binding site consists of amino acid residues Phe₁₄₈, Val₁₄₉, Asp₁₅₈.

3.1.2. Phylogenetic and molecular conservancy analysis of Per a 5 allergen

Phylogenetic analysis of Per a 5 with a set of homologous allergenic proteins was performed using Neighbor-joining algorithm in MEGA X tool. Reliability of generated tree was assessed by bootstrapping on 500 replicates and the phylogenetic tree can be broadly divided into three major clades (Fig. 1b). As per the phylogenetic tree, apart from delta class GSTs of *P. americana*, Per a 5 demonstrates significant evolutionary similarity with homologous allergens of *D. pteronyssinus*, Theta class GSTs of Humans, *T. aestivum*, *B. pendula* and fungal allergens *A. alternata* and *C. lunata*. GSTs belonging to *Aspergillus* and *E. maynei* (house dust mite) also belong to the same cluster, however are somewhat distant in comparison to the earlier mentioned GST allergens.

To gain an insight into evolutionary conservancy of Per a 5 with respect to other allergenic GSTs ConSurf analysis was performed. ConSurf utilizes Bayesian inference method to compute position specific conservation scores and identifies evolutionarily conserved amino acids having structural and functional significance. Per a 5 exhibits highly conserved residues corresponding to thioredoxin fold (βαβαββα) of GSTs. ConSurf estimation indicates towards fifteen highly conserved functional residues (Ser₁₁, Arg₁₅, Leu₁₈, Glu₄₄, Pro₅₀, Thr₅₃, Pro₅₅, Asp₅₉, Glu₆₆, Arg₈₆, Asp₈₈, Lys₉₁, Gly₁₀₁, Thr₁₅₅, Asp₁₅₈) exhibiting high surface accessibility and three conserved (Ala₂₁, Ser₆₇, Ile₇₀) residues of structural importance buried in protein structure. The putative GSH binding site in Per a 5 (G site, ranging from 10 to 67 aa in protein sequence) is most conserved as 63% residues exhibit conservation score above 5. In comparison, the substrate binding site (H site, ranging from 102 to 207 aa in protein sequence) possess 27 % conserved residues. The G site contains seven highly conserved residues (with evolutionary grade 9) as opposed to only two highly conserved residues in H site (Fig. 2). Since GSTs tend to form dimeric structures, to obtain a better understanding of conserved residues and their structural localization on three dimensional structure, Per a 5 was modelled as a homo dimer and conservation scores were projected onto it. Amino acid residues

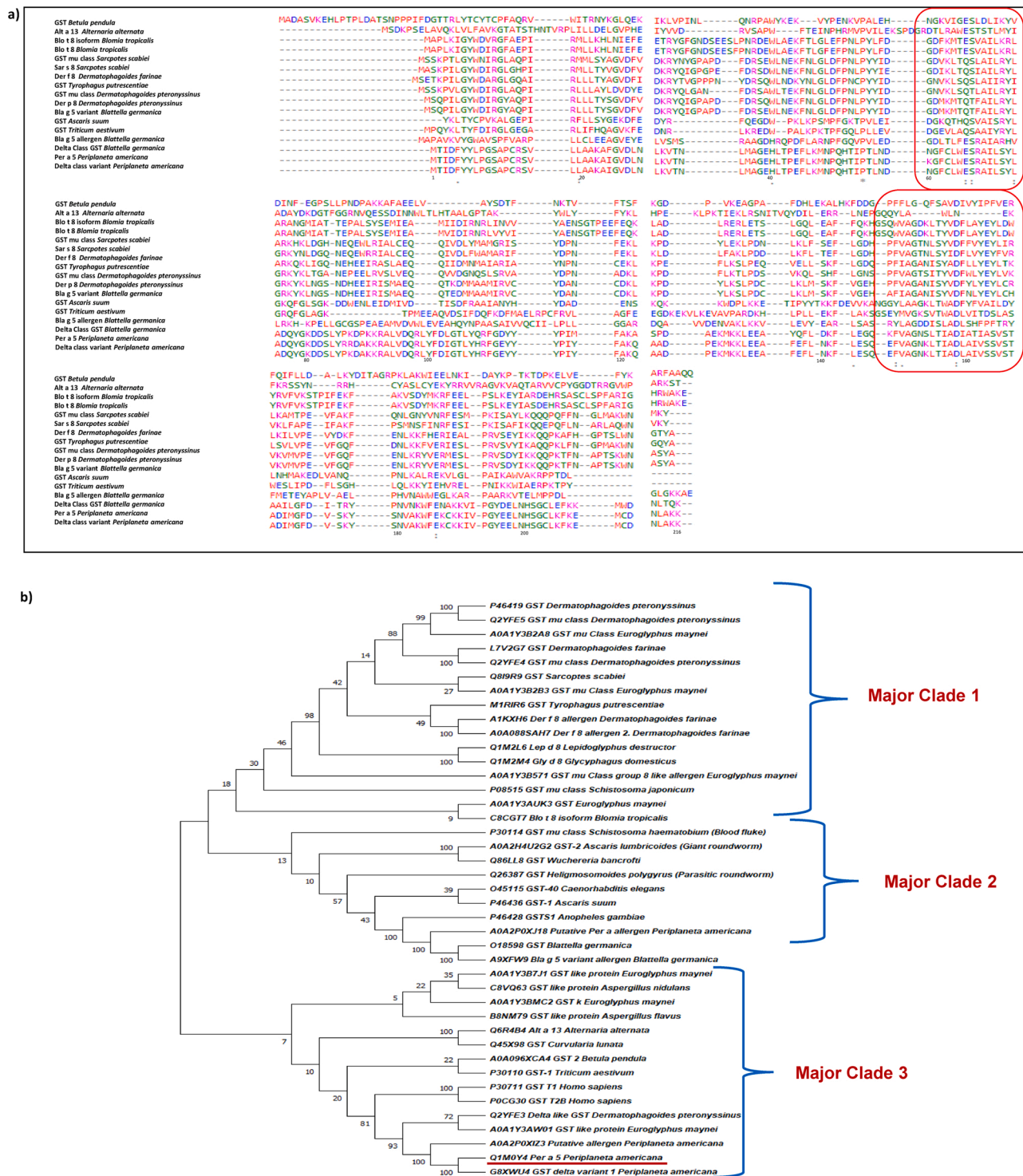


Fig. 1. Analysis of Evolutionary history of Per a 5: a) Multiple sequence alignment of Per a 5 with GST hits obtained by SDAP and Allergen Online databases, b) Phylogenetic tree: The evolutionary history was inferred using the Neighbor-Joining method in MEGA X. The optimal tree with the sum of branch length of 20.58484849 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Present analysis involved 41 amino acid sequences and all ambiguous positions were removed for each sequence pair.

occupying GST dimerization interface tend to exhibit high conservation scores.

3.2. Characterization of recombinant Per a 5 protein

Restriction digestion of the cloned construct with Eco R I and Xho I

revealed presence of 651 bp fragment (Supplementary Fig. 1). Per a 5 sequence in the construct was confirmed by gene sequencing. The protein was expressed in *E. coli* BL21 (λ DE3) cells and purified to homogeneity using Ni-NTA affinity chromatography. The yield of purified protein was approximately 1 mg/l of bacterial culture and resolved at 27 kDa on 12% SDS-PAGE (Fig. 3a). The recombinant protein was

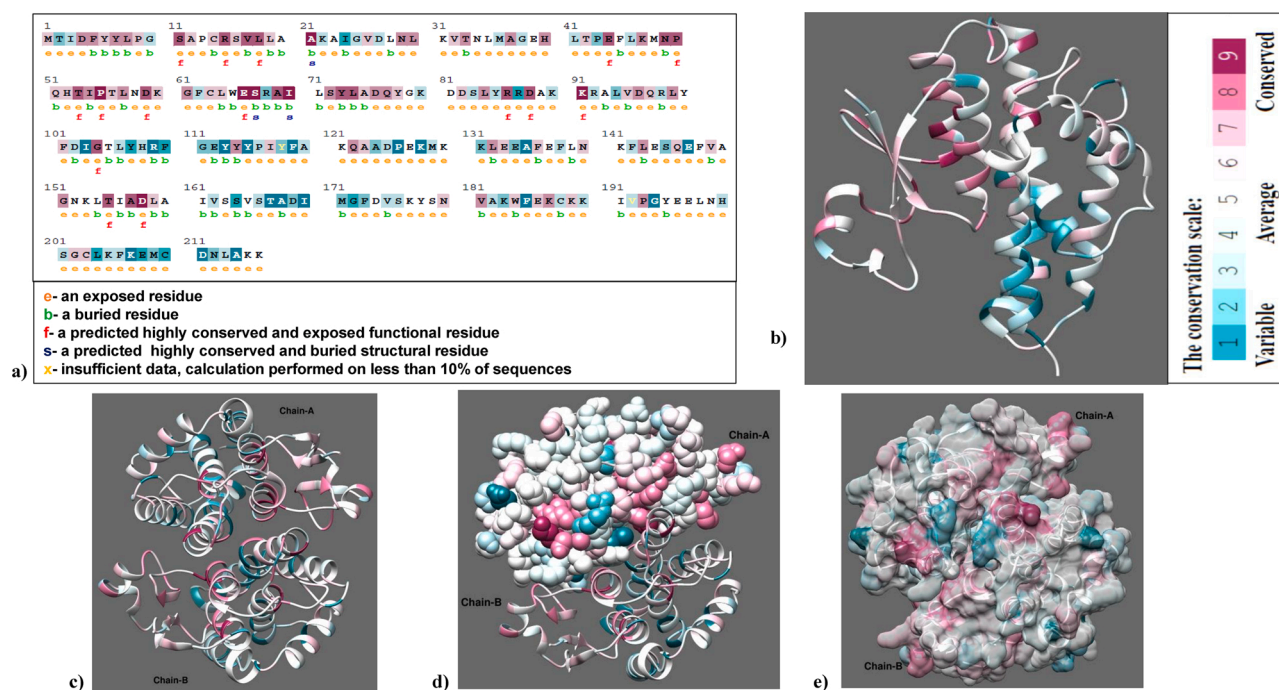


Fig. 2. Analysis of Evolutionary conservancy: Conservation analysis of Per a 5 protein with known GST allergens. a) ConSurf analysis of Per a 5 protein with homologous allergenic GSTs. b) Per a 5 structure depicting conservation status. c) Per a 5 homodimeric structure, monomers are designated as Chain A and Chain B, d) Per a 5 homodimer with Chain A presented as space filling model and Chain B in ribbon model displaying interacting residues at dimerization interface and e) surface model of Per a 5 homodimeric structure with 60 % transparency showing conservation status. The amino-acids are coloured by their conservation grades using the color-coding bar, with turquoise-through-maroon indicating variable-through-conserved. The 3-D model was visualised by UCSF Chimera 1.13.1.

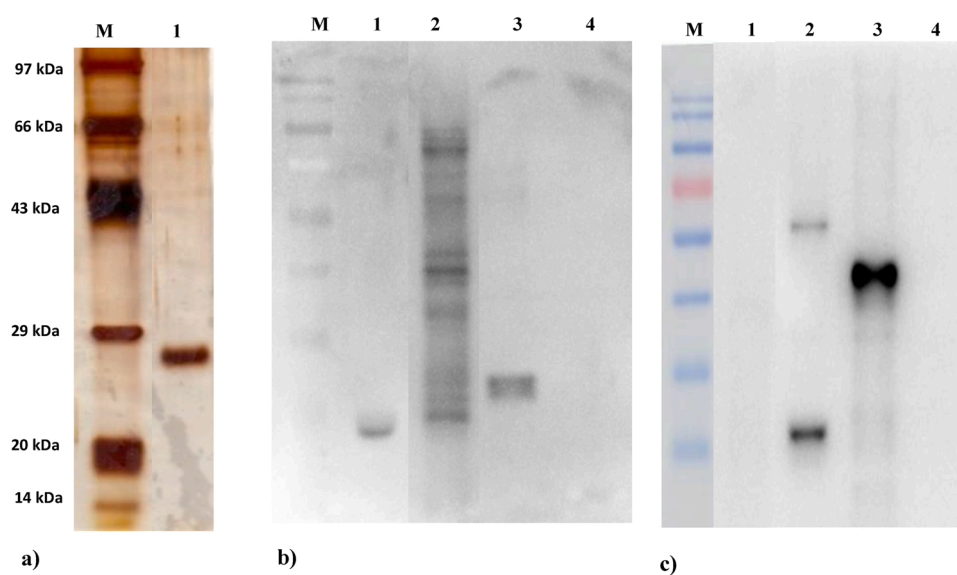


Fig. 3. Expression and biochemical characterization of rPer a 5: Per a 5 gene was cloned with C terminal 6X His tag into pET22b+ expression vector. The construct was transformed into *E. coli* BL21 DE3 cells and expressed using 1 mM IPTG inducer. rPer a 5 protein was purified using Ni-NTA affinity chromatography and resolved on 12% SDS PAGE. a) SDS PAGE profile of purified protein. The Per a 5 protein resolved at approx. 27 kDa size on 12 % SDS PAGE, b) Anti-GST blot (M: Marker Lane 1: Equine liver GST, Lane 2 Cockroach extract 10 µg, Lane 3 rPer a 5 500 ng, Lane 4: BSA 1 µg), c) Anti-Histidine blot (M: Marker Lane 1: Cockroach extract 10 µg, Lane 2: rPer a 5 500 ng, Lane 3: rCysteine Protease with His tag 1 µg and Lane 4: BSA 1 µg).

recognized by anti-GST and anti-Histidine antibody (Fig. 3b and c). GST activity assay was performed using CDNB, a broad specificity GST substrate. Equine liver GST exhibited 33.3 ± 0.497 nmol/min/µg of GST activity. rPer a 5 showed considerable activity with CDNB (0.2435 ± 0.0049 nmol/min/µg) and cumene hydroperoxide (23.01 ± 2.868 nmol/min), a substrate for glutathione peroxidases (Fig. 4).

3.3. Specific IgE binding of rPer a 5

The specific IgE binding of rPer a 5 protein was assessed by ELISA and Immunoblot using cockroach hypersensitive patient's sera. A total

of 15 patient's sera samples (SPT and ELISA positive to cockroach extract) were screened against rPer a 5 protein, out of which 7 patients (46.6 %) showed significant IgE reactivity. Sera samples from healthy individuals were used as negative control. OD value three times that of control sera was considered as ELISA positive result (Fig. 5b). Upon immunoblot analysis, 10 out of 15 patients showed IgE binding with rPer a 5 (66%) indicative of its IgE binding ability in cockroach hypersensitive patient's sera. (Fig. 5a). Among these, three patient samples (1, 9 and 10) exhibiting positivity on immunoblot showed low IgE binding in ELISA.

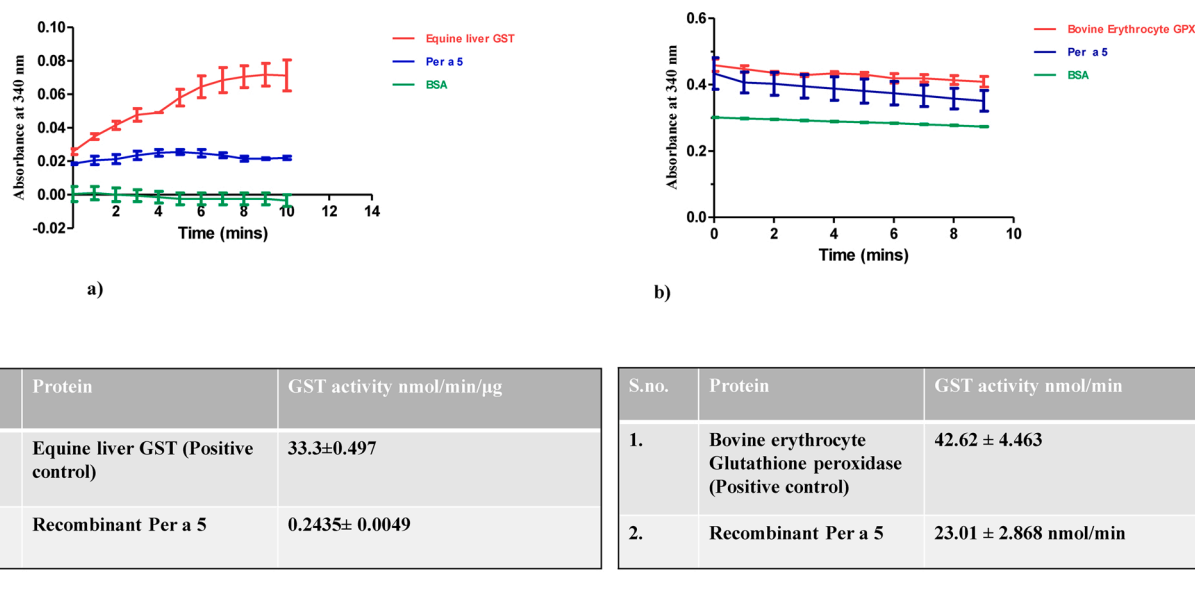


Fig. 4. Activity assay: Enzymatic activity analysis of rPer a 5 was performed with CDNB and cumene hydroperoxide. Purified rPer a 5 was reconstituted in sample buffer. The proteins were added in 96 well plate followed by addition of glutathione. Reaction was initiated by addition of CDNB and absorbance was measured at 340 nm every minute for 10 minutes. GST activity was calculated and plotted. Glutathione peroxidase activity was measured using Glutathione Peroxidase Assay kit (Cayman Chemical) following manufacturer's instructions. a) Kinetics of GST activity assay showing change in absorbance at 340 nm, b) Kinetics of Peroxidase activity of Per a 5 with Cumene Hydroperoxide indicated by decreasing absorbance at 340 nm and c) calculated GST activity of rPer a 5 with respective positive controls. Negative control used in both assays was BSA at 10 μg concentration. Calculated values of negative control are 0.033 nmol/min/ μg and 0.5858 ± 0.2038 nmol/min/ug for CDNB and cumene hydroperoxide respectively.

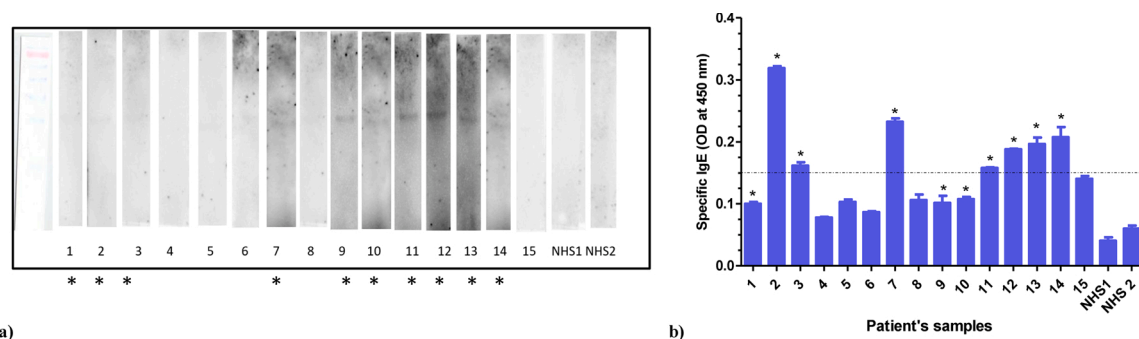


Fig. 5. IgE binding analysis of rPer a 5: a) The rPer a 5 protein was resolved on SDS PAGE and transferred onto nitrocellulose membrane. The membrane was blocked by 3% BSA and washed with PBST. The membrane was incubated with sera (1:20 diluted in 1% BSA in PBS) of specific IgE reactive positive patients overnight at room temperature. The membrane was washed with PBST and incubated with anti-Human IgE peroxidase (1:1000 dilution in 1 % BSA containing PBS). The membranes were again washed with PBST and developed using ECL substrate. Ten of the fifteen cockroach hypersensitive samples show IgE reactivity in immunoblot. b) ELISA of rPer a 5 protein with cockroach hypersensitive patient's sera: IgE binding ability of rPer a 5 was assessed by ELISA with serum samples of cockroach hypersensitive patients. The data is presented in graphical form depicting IgE binding as average ± SEM. Cut off value three times that of NHS i.e. 0.147 has been indicated by a dashed line on graph. Out of 15 cockroach hypersensitive patients 7 patients demonstrate IgE binding with rPer a 5. Sera samples exhibiting immunoblot positivity have been marked with *. Patient sample 1, 9 and 10 demonstrate immunoblot positivity but exhibit low IgE values in ELISA.

3.4. Basophil activation test

To assess biological ability of rPer a 5 protein in eliciting mediator release in effector cells, basophil activation test was performed by isolating peripheral blood mononuclear cells (PBMCs) from healthy volunteers followed by passive sensitization with patient's sera containing specific IgE. Flow cytometric evaluation of basophil specific marker CCR3 and activation marker CD203c revealed IgE dependent upregulation of basophil activation marker CD203c on CCR3+ basophils in PBMCs upon stimulation with CE and rPer a 5. The mean fluorescence intensity was calculated and plotted relative to unstimulated samples. A fold change of 2 and 1.75 was observed in CD203c levels in CE and Per a 5 challenged PBMCs (Fig. 6).

3.5. Inhibition studies

To investigate cross reactive potential of purified GST allergen, ELISA inhibition was performed with allergen extracts known to possess clinically relevant indoor allergens namely *C. lunata*, *A. alternata* and *D. pteronyssinus*. Inhibition studies were performed by incubating the pooled sera samples with increasing amounts of allergen extracts and their ability to inhibit Per a 5 specific IgE in sera samples was assessed by ELISA with solid phased bound rPer a 5 protein. All the extracts demonstrated dose dependent inhibition of bound rPer a 5 (Fig. 7). Maximum inhibition was demonstrated for cockroach extract (EC₅₀: 15.5 ng) followed by house dust mite (EC₅₀: 38.38 ng), *Alternaria alternata* (EC₅₀: 41.41 ng), and *Curvularia lunata* (EC₅₀: 61.66 ng). Inhibition results suggest strong cross reactive potential of Per a 5 with

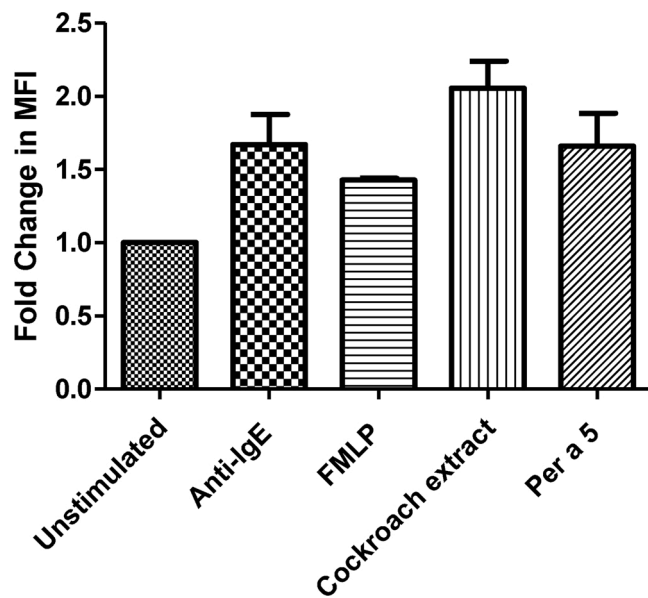


Fig. 6. Functional activity assay (Basophil activation Test): Basophil activation test was performed using passive sensitization protocol. PBMCs isolated from healthy volunteers were isolated and sensitized with pooled sera samples of cockroach hypersensitive patients. PBMCs were then challenged with cockroach extract and rPer a 5 for 30 min.. Anti-IgE and FMLP treated cells were taken as positive control. The cells were stained with PE anti-human CCR3 and APC-anti human CD203c and analysed by Flow cytometry. Basophils were selected as CCR3+ cells. Relative change in basophil activation is expressed as increase in CD203c levels in CCR3+ basophils.

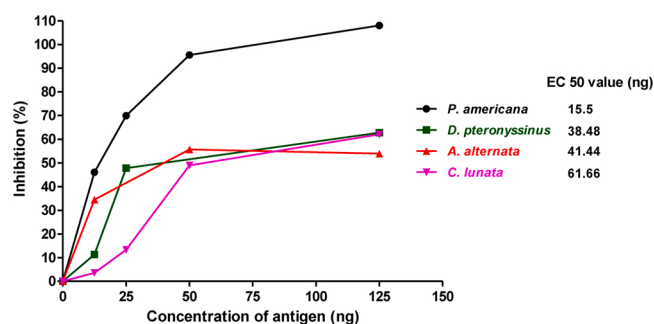


Fig. 7. Analysis of cross reactive potential: Inhibition studies were carried out by ELISA inhibition. rPer a 5 was coated on microtiter plates. The plates were blocked and pooled sera sample of cockroach allergic patients was incubated with increasing amounts of *Periplaneta americana*, *Dermatophagoides pteronyssinus*, *Alternaria alternata* and *Curvularia lunata* for 2 h at room temperature. Inhibited sera samples were added onto coated plates followed by developing ELISA as described previously. Percentage inhibition was calculated and plotted using GraphPad Prism.

GST homologs in dust mite and fungal extracts and are in close agreement with in silico determined evolutionary similarity.

4. Discussion

Cockroach allergens demonstrate a highly variable repertoire of allergenically relevant proteins, which is tremendously challenging to be comprehended by a limited set of two or three dominant allergens and thereby demonstrate a stark contrast to other potent allergen sources (such as dust mite and pollen) comprising a few highly immunodominant allergens. This complexity is further compounded by geographical and climatic variations and presence of co-sensitizing and cross-reactive allergens in the milieu. The present study was undertaken

to characterise delta class GST allergen Per a 5 of *P. americana* and to evaluate its allergenic relevance and cross reactive potential.

A combination of in silico tools were employed to determine potentially similar and cross reactive allergens of Per a 5. Sequence homology search for Per a 5 revealed it to be similar to clinically relevant GST homologs from mite, helminth and fungal sources. Conventionally, proteins demonstrating sequence identity above 50% are more likely to demonstrate cross reactivity however recent studies have demonstrated greater importance of protein conformational structures and phylogenetic relatedness in influencing cross reactivity (Aalberse, 2000; Acevedo and Caraballo, 2011; Sharp et al., 2014; Nugraha et al., 2019). A notable example is GST from helminth *Wuchereria bancrofti* which demonstrates significant cross reactivity with Bla g 5, even at low sequence identity of 27.9% (Santiago et al., 2012). Analysis of evolutionary similarity may offer an advantage over traditional sequence based approaches and might offer an explanation to deviations observed. Therefore, we sought to envisage phylogenetic relationship of Per a 5 with homologous GSTs belonging to different allergen sources. Phylogenetic tree constructed using MEGA X software clearly demonstrates Per a 5 to cluster with allergens from dust mite (*E. maynei* and *D. pteronyssinus*) and fungal allergens (*A. alternata*, *C. lunata*). Per a 5 demonstrates close evolutionary similarity with allergens from birch GST2 from *B. pendula* and wheat allergen *T. aestivum*. GST allergen from birch (*B. verrucosa*) has been shown to cross react with *S. mansoni* GST owing to shared glycan epitopes (Igetei et al., 2018). Helminth GSTs (from *W. bancrofti*, *A. suum*) tend to cluster with insect GSTs including Bla g 5 which is in accordance with earlier studies (Fig. 1b) (Santiago et al., 2012). Interestingly, theta class GST from human (GST-T1) also demonstrated evolutionary similarity with Per a 5. A thorough analysis of potential cross reactivity at linear or discontinuous epitope level is warranted given the essential function and ubiquitous distribution of GST-T1 in humans. ConSurf analysis indicate towards clusters of amino acids with high degree of evolutionary similarity in N terminal region, especially involved in dimerization of monomeric Per a 5 protein. A majority of these residues (15 residues) have been predicted as highly conserved surface accessible residues with critical role in protein functionality, while three have been predicted to be highly conserved residues of structural importance occupying buried regions of protein.

Analysis of immunological cross reactivity cannot solely be ascribed by sequence homology studies, we further sought to examine the allergenic relevance and cross reactive potential of rPer a 5. Per a 5 gene was sub-cloned and expressed in *E. coli* and purified by affinity chromatography. The obtained protein resolved at 27 kDa in SDS-PAGE. To determine its functional activity, activity assay was performed with substrates CDNB and cumene hydroperoxide. CDNB is a broad specificity GST substrate while cumene hydroperoxide is a substrate for Glutathione peroxidases (GPOX). Majority of insects lack specialized enzyme systems for protection against reactive oxygen species and rely on GSTs for peroxidase activity as well. Purified rPer a 5 demonstrated significant activity with CDNB and a pronounced activity with cumene hydroperoxide. Earlier, delta class GST allergen (BgGSTD1) from *B. germanica* has been shown to demonstrate high peroxidase activity (Ma and Chang, 2007). The sigma class GST from german cockroach, Bla g 5 is also a potent allergen, however its delta class counterpart is less relevant (Arruda et al., 1997; Ma and Chang, 2007). Immunological analysis of rPer a 5 demonstrated it to be an important allergic sensitizer in ELISA and immunoblot with 46.6 and 65% IgE reactivity. The differences may be due to allergen adsorption (on well or membrane) and its effect on availability of epitopic regions in serum IgE. Deviation in IgE binding data have been observed in a few studies recent being with GRP allergen of Japanese apricot and Der f 23 allergen of mites (Inomata et al., 2017; He et al., 2019). Further immunological analysis of rPer a 5 was performed with Basophil activation test (BAT). rPer a 5 upregulated the expression of CD203c on surface of CCR3+ basophils in human PBMCs signifying its biological ability to bind and crosslink IgE bound on Fcε receptors on basophils.

House dust mite, cockroach and molds are major allergenic triggers in tropical and sub-tropical regions especially in indoor urban areas (Caraballo et al., 2016). Earlier in silico data suggests Per a 5 as a potentially cross reactive allergen with GSTs present in fungal and dust mite allergen extracts. Previously, a comparative analysis of a variety of fungal extracts demonstrated GSTs to be majorly cross reactive proteins (Shankar et al., 2005). Also, the dust mite GST allergen Der p 8 has been shown to be cross reactive with cockroach GST (Huang et al., 2006). Analysis of helminth allergenic components revealed GST to be a clinically relevant allergen cross reactive with mite allergens (Acevedo et al., 2013). Therefore, we sought to evaluate cross reactivity of Per a 5 by means of ELISA inhibition studies with extracts of *D. pteronyssinus*, *A. alternata* and *C. lunata*. Per a 5 demonstrated high cross reactivity with dust mite allergen, followed by fungal allergens (*A. alternata* and *C. lunata*). These results are in agreement with in silico observed evolutionary phylogenetic analysis.

Discerning the cross reactive potential of allergens is challenging given the diverse roles played by multitude of factors including co-sensitizing and cross sensitizing allergens in environment and presence of shared glycan epitopes. Birch GST, house dust mite chitinase (Der f 15) and cockroach arylphorin (Per a 3) have already been demonstrated to be cross reactive with helminth counterparts owing to CCDs (El-Faham et al., 2020; Igetei et al., 2018). Comparative analysis of cross reactivity amongst GST allergens in temperate regions indicate low in vitro cross reactivity and unique epitopic regions in Bla g 5 and Der p 8, however simultaneous exposure to fungal, mite and helminth allergens in tropical regions may exacerbate the clinical manifestations (Mueller et al., 2015). Reports have emerged regarding the allergenic relevance of Per a 5, as it has been identified as a minor and major allergen in USA and Thailand respectively, underscoring the impact of environmental factors on allergic sensitization (Wei et al., 2014; Sook-rung et al., 2018). Our study reinforces the importance of *P. americana* GST allergen Per a 5, as a cross reactive allergen. The GST allergen may possess clinical relevance in countries with indiscriminate use of insecticides and higher exposure to cross reactive and cross sensitizing sources (fungal spores and dust mite) and helminth infections (Acevedo et al., 2013; Maizels, 2020). The presence of co-sensitizing cross reactive allergens in tropical regions may also hinder accurate diagnosis and enhance the risk of associated clinical manifestations. A combination of allergenically relevant recombinant proteins may be utilised for efficient diagnosis and allergy therapeutics.

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Ethics approval

The study was approved by Institutional Human Ethics Committee.

CRedit authorship contribution statement

Swati Sharma: Conceptualization, Methodology, Writing - original draft. **Bharti Arora:** Methodology, Writing - review & editing. **S.N. Gaur:** Resources, Supervision. **Naveen Arora:** Conceptualization, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

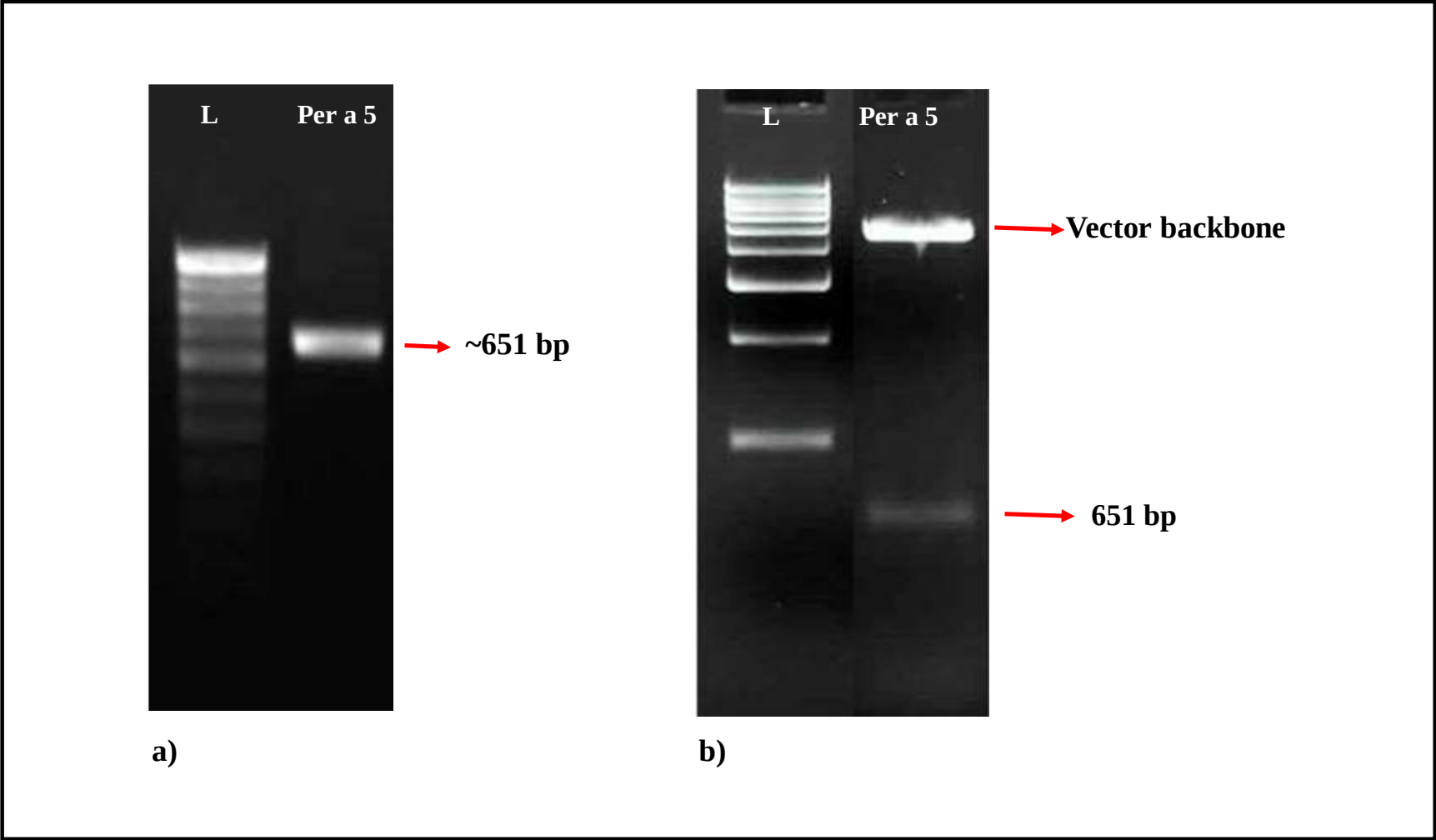
Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2021.01.026>.

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Supplementary Figure 1.



Supplementary Table 1.

Per a 5 similarity with GST allergens

S.no.	Protein	Uniprot ID	Sequence Similarity (%)	Sequence Identity (%)	E value
1.	Glutathione S transferase class delta variant 1 [<i>Periplaneta americana</i>]	G8XWU4	99.5	98.6	5.9e-098
2.	Delta class glutathione S-transferase [<i>Blattella germanica</i>]	A7E1B7	94.9	80.6	1.2e-082
3.	Glutathione transferase mu class Yv5004H11 [<i>Sarcoptes scabiei</i>]	Q2YFF0	54.7	28.9	4.2e-006
4.	Der f 8 allergen [<i>Dermatophagoides farinae</i>]	A0A088SAH7	58.2	27.6	3.1e-005
5.	Glutathione transferase [<i>Triticum aestivum</i>]	Q8GTC0	52.3	24.9	3.1e-005
6.	Glutathione-S-transferase Allergen, Alt a 13	Q6R4B4	60.5	23.5	7.4e-005
7.	Der f 8 allergen, partial [<i>Dermatophagoides farinae</i>]	A1KXH6	54.2	25.0	0.00052
8.	Glutathione S-transferase [<i>Sarcoptes scabiei</i>]	A0A132ALW8	56.9	22.3	0.00052
9.	Glutathione S-transferase 1; sigma class GST from <i>Ascaris suum</i>	P46436	56.1	26.5	0.00059
10.	Glutathione S-transferase [<i>Betula pendula</i>]	A0A096XCA4	55.8	24.0	0.0013
11.	Tyr p 8 allergen [<i>Tyrophagus putrescentiae</i>]	M1RIR6	57.6	23.0	0.012
12.	Glutathione transferase mu class [<i>Dermatophagoides pteronyssinus</i>].	Q2YFE5	56.8	20.1	0.024
13.	Major allergen Der p 8 [<i>Dermatophagoides pteronyssinus</i>]	P46419	56.8	19.4	0.028
14.	Glutathione S-transferase Blo t 8 isoform [<i>Blomia tropicalis</i>]	C8CGT7	53.0	25.2	0.042
15.	Blo t 8 allergen [<i>Blomia tropicalis</i>]	A1KXG6	52.3	24.5	0.081
16.	Bla g 5 variant	A9XFW9	50	25.4	
17.	Bla g 5 allergen	O18598		24.2	0.13

Patient No.	Age/ Sex	Reaction in SPT (mm)	Specific IgE OD	Ratio patient OD/ avg NHS (0.049)
1	34/M	++++	0.097	1.97
2	32/M	++	0.316	6.44
3	29/M	++++	0.156	3.71
4	23/M	++++	0.079	1.61
5	50/M	++++	0.099	2.02
6	36/F	+++	0.085	1.73
7	20/F	+++	0.238	4.85
8	26/M	+++	0.115	2.34
9	20/M	+++	0.090	1.83
10	31/M	++++	0.111	2.26
11	36/M	+++	0.159	3.24
12	23/F	+++	0.187	3.81
13	36/M	++++	0.186	3.79
14	35/M	++	0.224	4.57
15	38/M	++	0.145	2.9
16	NHS1		0.035	-
17	NHS2		0.065	-

Supplementary Figure legends

Supplementary Figure 1. Cloning of Per a 5 gene: Total RNA was isolated from freeze dried cockroach powder and cDNA was prepared. Per a 5 gene was PCR amplified using gene specific primers and the amplified PCR product was run on 1% Agarose gel. Per a 5 gene was cloned in pET 22b+ expression vector using Eco R1 and Xho 1 restriction sites. The restriction digested vector and Per a 5 were ligated by T4 DNA ligase and positive colonies were screened. The cloned construct was sequenced for confirmation a) Gel image of Per a 5 PCR product at approx 651 bp. b) Restriction digestion of positive colony demonstrates insert at 651 bp. L: 100 bp ladder

Supplementary Table 1. Per a 5 demonstrates similarity with insect GSTs: Per a 5 protein sequence was submitted to databases SDAP and AllergenOnline. Allergens demonstrated highest sequence similarity and identity are presented in tabular form.

Supplementary Table 2. ELISA of rPer a 5 protein with cockroach hypersensitive patient's sera: IgE binding ability of rPer a 5 was assessed by ELISA with serum samples of cockroach hypersensitive patients. Out of 15 cockroach hypersensitive patients 7 patients demonstrate IgE binding with rPer a 5. NHS: Normal Human sera. ELISA positive samples have been indicated with maroon color.