

Rationally designed hypoallergenic mutant variants of the house dust mite allergen Der p 21

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ABSTRACT

Background: Allergic diseases figure among the most common immune-mediated diseases worldwide, affecting more than 25% of the world's population. Allergic reactions can be triggered by house dust mite (HDM) allergens, of which the so-called group 21 of allergens is considered as clinically relevant.

Methods: Herein, we used a structural bioinformatics and immunoinformatics approach to design hypoallergenic mutant variants of the Der p 21 allergen of *Dermatophagoides pteronyssinus*, which were then recombinantly expressed in bacteria and tested for their IgE-reactivities. For this, we scanned the wild-type Der p 21 protein for all possible single amino acid substitutions in key IgE-binding regions that could render destabilization of the major epitope regions.

Results: Four main substitutions (D82P, K110G, E77G, and E87S) were selected to build mutant variants of the Der p 21 allergen, which were produced in their recombinant forms; two of these variants showed reduced reactivity with IgE. Molecular dynamic simulations and immune simulations demonstrated the overall effects of these mutations on the structural stability of the Der p 21 allergen and on the profile of immune response induced through immunotherapy.

Conclusions: When produced in their recombinant forms, two of the Der p 21 mutant variants, namely proteins K110G and E87S, showed significantly reduced IgE reactivities against sera from HDM-allergic individuals ($n = 20$; $p < 0.001$).

General significance: This study successfully translated a rational *in silico* mutagenesis design into low IgE-binding mutant variants of the allergen rDer p 21. These novel hypoallergens are promising to compose next-generation allergen-immunotherapy formulations in near future.

1. Introduction

The most common manifestations of allergic diseases are immunoglobulin E (IgE)-mediated Type I hypersensitivity reactions [1,2], which affect a significant proportion of the global population [3]. One of the main risk factors to the development of Type I hypersensitivity is the sensitization to allergens from the house dust mites (HDM) [4–7]. *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are the major

sources of HDM allergens and they are widespread in many regions of the globe, triggering allergic symptoms [6,8,9].

Among the *Dermatophagoides pteronyssinus* allergens, Der p 21 was characterized as a new important allergen, with a high capacity for binding to IgE antibodies and high allergenic activity [10,11]. Pulsawat et al. 2014 suggested that Der p 21 can stimulate the production of IL-8 in airway epithelial cells through TLR2-dependent signaling, similarly to the mechanism described for Der p 2 (a major allergen of

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D. pteronyssinus) [11]. The prevalence of reactivity to Der p 21 has also been investigated by different studies: 25% of subjects in a cohort of HDM-allergic patients in Thailand showed IgE reactivity to this allergen [11]; in Austria, the reported reactivity was 26% [10]; besides, similar percentages of IgE reactivity (ca. 20%) were also reported in studies from Singapore [12] and Germany [13]. The Der p 21 sequence has similarities with those of group 5 allergens, but does not show significantly high cross-reactivity. The sequence alignments have shown that Der p 21 and Blo t 5, from the mite *Blomia tropicalis*, have an amino acid (AA) sequence identity of ca. 40% [14], while Der p 21 and Der p 5 have ca. 31% identity [15]. Sequence studies also revealed that Der p 21 and Blo t 21 share ca. 41% of AA sequence identity [15]. These features indicate that Der p 21 is a potential candidate not only for IgE-mediated allergy diagnosis, but also for allergen-specific immunotherapy (AIT) against allergy triggered by *D. pteronyssinus*.

AIT is a traditional treatment for patients suffering from IgE-associated allergy [16] and can be more effective than pharmacotherapy and immunoreactive biologicals in reducing allergy symptoms. The AIT approach takes advantage of the patient's own immune system, and it is the only treatment that shows disease-modifying effects and prevents the progression of severe symptoms [1,3,17]. However, traditional AIT based on crude mite extracts has important limitations due to the potential of inducing severe and systemic side effects, which require up-dose schedules and multiple administrations [3,18,19].

To circumvent these drawbacks, advancements in molecular allergen characterization led to the development of new forms of AIT based on recombinant purified proteins and hypoallergenic peptide derivatives [18,20]. In the hypoallergenization process, allergen derivatives can be produced by identifying the amino acid sequence determinants that are essential to IgE reactivity, and these regions are mutated or excluded at the genetic level, before recombinant protein production. Structural bioinformatics and immunoinformatics tools are generally employed to seek and rationally modify these protein regions in order to maintain the IgG reactivity, while decreasing IgE binding [3,21].

In this study, hypoallergenic recombinant molecules derived from the wild-type (wt) Der p 21 allergen were designed by using a dedicated structural bioinformatics analysis pipeline. Our approach involved *in silico* mutagenesis of the target allergen, allowing for the rational design of hypoallergenic variants. Prior knowledge of the allergen's three-dimensional structure and the location of B cell epitopes are required, enabling a targeted modification of these epitopes, but maintaining the immunogenicity of the allergen. We found that two mutant variants of Der p 21, namely K110G and E87S, presented significant decrease in IgE reactivities when tested with sera of HDM-allergic subjects, demonstrating their potential to compose hypoallergenic AIT formulations.

2. Materials and methods

2.1. Structural bioinformatics and *in silico* mutagenesis of the Der p 21 allergen

The structural bioinformatics pipeline and the *in silico* design of the of Der p 21 hypoallergenic mutant variants are summarized in the Supplementary Material Fig. S1 and described in the subsequent items.

2.1.1. Sequence retrieval and analyses of protein domains and post-translational modifications

Database entries containing information about Der p 21 were searched in the Allergome relational database (<http://www.allergome.org/>) [22]. Allergome entries contain links to the sequences in the UniProtKB protein database (<http://www.uniprot.org/>) [23]. Domain analyses were performed with the Hmmer Profile Search (<http://hmmerr.org/>) against the pfam database (<http://pfam.xfam.org/>) [24]. Domain prediction was used as a means to try to get further insights on the epitope positions within the Der p 21 allergen, i.e. whether or not major epitopes co-localize with predicted protein domains.

2.1.2. Protein tertiary structure prediction, refinement, and validation

Der p 21 wild-type sequences were submitted to the Robetta protein tertiary structure prediction server, using standard parameters (*de novo* modeling and homology modeling) (<http://rosetta.bakerlab.org/>) [25]. Refinement was carried out with FoldIt Standalone [26], which uses a RosettaCM algorithm to refine the protein sidechains and backbone based on free energy score (kcal/mol). Protein tertiary structure validation was then performed using the QMEANDisCo server (<https://swissmodel.expasy.org/qmean/>) [27] (Supplementary Table S1). 3D-structures visualization was performed with FoldIt and PyMOL Molecular Graphics System (<https://pymol.org/>).

2.1.3. Prediction of B-cell epitopes

Epitopes recognized by B cells were predicted on multiple prediction tools in order to build a consensus and a robust result. CBTope (<http://osddlinux.osdd.net/raghava/cbtope/submit.php>) [28] analysis were performed with a cut-off value for the SVM Threshold of -0,3 (default), because at this value, sensitivity and specificity were found equal during the development of the software. ElliPro (<http://tools.iedb.org/ellipro/>) [29] analyses were performed selecting the minimum score of 0.6 (default is 0.5) and maximum distance of 6 Angstroms (default). Both parameters guarantee good sensitivity and, particularly, good specificity. BCPRED (<http://crdd.osdd.net/raghava/bcpred/>) [30] analyses were performed using parameters of Antigenic Propensity by Kolaskar et al., 1990 [31]. DiscoTope (<https://services.healthtech.dtu.dk/service.php?DiscoTope-2.0>) [32] analyses were performed with the threshold for epitope prediction being set at -1.0. At this value, sensitivity was described as 0.30 and specificity as 0.85. Chimera software (<https://www.cgl.ucsf.edu/chimera/>) was used for the visualization of epitopes.

2.1.4. Predictions of T-cell epitope cleavage sites

MHC-II processed ligands prediction analyses were performed on the IEDB Tools MHCII-NP (<http://tools.iedb.org/mhciiinp/>) [33] server using sequences from Der p 21 wild-type and Der p 21 with all substitutions selected together. Comparison between Der p 21 and the mutants with 4 substitutions was performed to analyze the difference between the MHC II predicted peptides between the variants.

2.1.5. *In silico* mutagenesis of Der p 21

Amino acid residues with the highest scores on the B-cell epitopes prediction servers results were selected to build a consensus and undergo *in silico* mutagenesis. The positions 77 (glutamic acid), 82 (aspartic acid), 87 (glutamic acid), and 110 (lysine) of the Der p 21 wt allergen were selected to a following a scan for the most destabilizing mutations, performed with the MAESTROweb server (<https://biwww.che.sbg.ac.at/maestro/web/>) [34]. A manual curation was performed to make sure that mutagenesis would not occur in sites that could lead to a complete disruption in the secondary structure or, consequently, in the tertiary structure of the original protein, such as, for example, proline in an alpha helix. The results of the *in silico* mutagenesis performed with MAESTROweb were then validated using the FoldIt Standalone program. Also, with the FoldIt *in silico* mutagenesis was performed using the 'mutate' tool. After each substitution, a round of protein structure stabilization was carried in order to submit the protein structure to the effects of the aminoacidic change.

Finally, combinations were made between the most destabilizing mutations identified (E77G, D82P, E87S and K110G) for the production of Der p 21 wt variants: P1.D82P (Asp82Pro substitution), P2.K110G (Asp82Pro + Lys110Gly substitution), P3.E77G (Asp82Pro + Lys110Gly + Glu77Gly substitution) and P4.E87S (Asp82Pro + Lys110Gly + Glu77Gly + Glu87Ser substitution) (Table 1).

2.1.6. Molecular dynamics simulation

To evaluate the structural stabilities of the Der p 21 mutant variants in comparison to the wild-type allergen, we performed a molecular

Table 1

Predictions of the most destabilizing amino acid (AA) substitutions in the Der p 21 allergen and characteristics of the mutant derivatives constructed in this study.

AA position ^a	Original AA	MAESTRO predictions ^b			FoldIt validation ^b	Mutant derivatives (AA substitutions)
		Suggested substitution	$\Delta\Delta G$ (kcal/mol)	Cpred ^c	$\Delta\Delta G$ (kcal/mol)	
82	Asp (D)	Pro (P)	1.275	0.904	11.641	P1.D82P (Asp82Pro)
110	Lys (K)	Gly (G) ^d	0.881	0.912	5.785	P2.K110G (Asp82Pro + Lys110Gly)
77	Glu (E)	Gly (G)	2.563	0.815	3.472	P3.E77G (Asp82Pro + Lys110Gly + Glu77Gly)
87	Glu (E)	Ser (S)	2.036	0.844	2.910	P4.E87S (Asp82Pro + Lys110Gly + Glu77Gly + Glu87Ser)

^a Relative to Der p 21 wild-type sequences retrieved from Allergome DB.^b Starting Gibbs free energy value = -176.553 kcal/mol.^c Confidence estimation of the calculated $\Delta\Delta G$. The values vary between 0.0 and 1.0, where 1.0 corresponds to a perfect prediction accuracy.^d Firstly predicted as Pro (P) but discarded due to potential alpha-helix break.

dynamics simulation experiment. Simulations were carried out by using the GROMACS 2020.3 package [35]. CHARMM36 force field [36] was chosen to perform analysis. Proteins were solvated in a dodecahedron box with TIP3P water molecules. Na⁺ and Cl⁻ ions were added to the system to achieve a neutral charged system. Electrostatic interactions were calculated using the Particle Mesh Ewald for long-range electrostatics method [37]. V-rescale temperature coupling (modified Berendsen) was used to control temperature (310 Kelvin). Berendsen pressure coupling [38] was used to control pressure (1.0 bar). The systems were subjected to unrestrained molecular simulation for 100 ns. Comparative analyses of structural deviations in wild-type and mutant structures were performed. RMSD and RMSF analyses were carried out using GROMACS tools. Graphs were plotted using MATLAB 2020 [39].

2.1.7. Immune simulation analysis

To predict the immune response induced by Derp21 (wt) or by the mutant proteins, we conducted an immune stimulation using C-ImmSim server (<http://www.cbs.dtu.dk/services/C-ImmSim-10.1/>) [40]. For simulation, two injections containing 1000 vaccine proteins each were given at intervals of one week. Time steps are set at 1 and 21 (each time step is 8 h in real life, and time step 1 is the time of injection = 0). The simulation steps have been modified to 1050, and the other parameters were kept as default.

2.2. Design and construction of recombinant plasmids

Table 2 describes all synthetic gene fragments and plasmids constructed in this study. Synthetic genes encoding rDer p 21 wt and the four mutant variants were obtained from Eurofins (Luxemburg), and then cloned using the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, EUA). The resulting plasmids were transformed into *E. coli* XL1Blue stain and recombinant clones were cultivated at 37 °C, in Luria-Bertani (LB) broth, with kanamycin. Plasmids were recovered using the Nucleospin plasmid QuickPure kit (Macherey Nagel Co., Düren, Germany), the gene fragments were digested with *SapI* and then subcloned in the expression vector pOPT 2.0 (Table 2) (Supplementary Fig. S2), using standard protocols. Confirmation was performed by digestion with the restriction enzymes *EcoRI* and *PstI* (Promega, Madison, WI, USA) and analysis in a 1% agarose gel.

2.3. Expression, purification, and analysis of recombinant Der p 21 and mutant variants

The expression vectors constructed in this study (Table 2) were transformed into chemically competent *E. coli* BL21(DE3) strains. The cells were grown at 37 °C overnight in LB-agar plates supplemented with 34 µg.mL⁻¹ of chloramphenicol. Confirmed clones were incubated in 10 mL LB broth (34 µg.mL⁻¹ Cm) and induction of the target recombinant proteins was performed with 1 mM IPTG (Invitrogen, CA, California, EUA), at an O.D._{600nm} = 0.6. Cells were then incubated at 37 °C for up to

Table 2

List of synthetic gene fragments, plasmids and bacterial strains used in this study.

	Characteristics	Source
Synthetic gene fragments		
<i>derp21wt</i>	387 bp; optimized synthetic gene fragment coding for the recombinant Der p 21 allergen.	This study
<i>mut1</i>	387 bp; optimized synthetic gene fragment coding for the Der p 21 mutant variant P1.D82P.	This study
<i>mut2</i>	387 bp; optimized synthetic gene fragment coding for the Der p 21 mutant variant P2.K110G.	This study
<i>mut3</i>	387 bp; optimized synthetic gene fragment coding for the Der p 21 mutant variant P3.E77G.	This study
<i>mut4</i>	387 bp; optimized synthetic gene fragment coding for the Der p 21 mutant variant P4.E87S.	This study
Strains		
<i>E. coli</i> XL1Blue	<i>endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F' [:: Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+)</i>	Stratagene
<i>E. coli</i> BL21 (DE3)	B F- ompT gal dcm lon hsdSB(rB-mB-) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]-K12(ΔS)	Stratagene
Plasmids		
pCR-Blunt II-TOPO	ColE1/ Kan ^R , Neo ^R ; <i>lacZalpha</i> , <i>ccdB</i>	Invitrogen
pTP1: <i>derp21wt</i>	ColE1/ Kan ^R , Neo ^R ; fragment encoding the recombinant Der p 21 allergen (rDer p 21), flanked by <i>SapI</i> sites.	This study
pTP2: <i>mut1</i>	ColE1/ Kan ^R , Neo ^R ; cloned fragment encoding the Der p 21 mutant variant P1.D82P, flanked by <i>SapI</i> sites.	This study
pTP3: <i>mut2</i>	ColE1/ Kan ^R , Neo ^R ; cloned fragment encoding the Der p 21 mutant variant P2.K110G, flanked by <i>SapI</i> sites.	This study
pTP4: <i>mut3</i>	ColE1/ Kan ^R , Neo ^R ; cloned fragment encoding the Der p 21 mutant variant P3.E77G, flanked by <i>SapI</i> sites.	This study
pTP5: <i>mut4</i>	ColE1/ Kan ^R , Neo ^R ; cloned fragment encoding the Der p 21 mutant variant P4.E87S, flanked by <i>SapI</i> sites.	This study
pOPT2.0	pUC ori/ Cm ^R ; Expression vector containing an optimized T7 promoter, strong RBS, fragment encoding GFP, 6× His-tag, T7 term. <i>SapI</i> -mediated scarless cloning.	This study
pOPT: <i>derp21wt</i>	pOPT2.0 containing a cloned gene fragment encoding the recombinant Der p 21 allergen (rDer p 21).	This study
pOPT: <i>mut1</i>	pOPT2.0 containing a cloned gene fragment encoding the Der p 21 mutant variant P1.D82P.	This study
pOPT: <i>mut2</i>	pOPT2.0 containing a cloned gene fragment encoding the Der p 21 mutant variant P2.K110G.	This study
pOPT: <i>mut3</i>	pOPT2.0 containing a cloned gene fragment encoding the Der p 21 mutant variant P3.E77G.	This study
pOPT: <i>mut4</i>	pOPT2.0 containing a cloned gene fragment encoding the Der p 21 mutant variant P4.E87S.	This study

20 h. For recombinant protein purification, centrifuged bacterial pellets were collected at different time points during the induction protocol. Pellets were resuspended with 50 mM phosphate buffer, with or without 6 M urea (pH 8.0). Resuspended pellets were sonicated thrice, for 30 s at 50 Hz. His-tagged recombinant proteins were purified using the MagneHis™ Protein Purification system (Promega, Madison, WI, EUA), following manufacturer's instructions. Recombinant proteins were resolved in 12% SDS-PAGE and confirmed by Western blot using mouse anti-His IgG (GE Healthcare, 1:4000). Protein concentration was measured with the Qubit™ Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, EUA).

2.4. Sera from HDM-allergic patients

In this study, two sets of sera were used. The first group of donors was composed of sensitized individuals, from both genders, aged ≥ 18 years, and living in the city of Salvador, Brazil. The second set of sera was obtained from individuals enrolled in Asthma and Allergic Rhinitis Control Program in Bahia (ProAR) [41]. The allergy diagnosis, in both cases, was based on allergy symptoms (rhinitis, conjunctivitis, and/or asthma), positive skin prick test to *Dermatophagoides pteronyssinus*, and serum levels of IgE anti-*D. pteronyssinus* using immunoCAP ($> 0,7$ kUA/L). Sera from nonallergic individuals were used as control. The study was approved by the Ethics Committee on Research of the Faculty of Medicine of the Federal University of Bahia (CAAE 45376814.0.0000.5577). Blood samples were taken after informed consent was obtained from patients.

2.5. IgE reactivities of recombinant Der p 21 wt and of the mutant variants

Firstly, a Dot blot assay was performed to analyze the IgE reactivity to the Der p 21 wt and the mutant variants. For this, 2.5 μg of each recombinant protein was applied to a nitrocellulose membrane (GE Healthcare, Waukesha, WI, USA). Likewise, a crude extract of *Dermatophagoides pteronyssinus* and a purified recombinant Der p 2 protein were used as positive controls. After 1.5 h incubation with the antigens, membranes were blocked for 2 h at room temperature using a 3% skimmed milk and 5% tween-20 PBS solution. Then, membranes were incubated with pools of sera (1:5 diluted) at 4 °C, for 16 h. A sera pool of five samples from sensitized patients was used, and another pool of five non-allergic patients was used as control. Following sera incubation, the membranes were incubated with a biotinylated anti-human IgE antibody (1:500, BD Biosciences, San Jose, CA) for 1 h at room temperature and then incubated with Streptavidin-HRP (1:2500, BD Biosciences, San Jose, CA) for 45 min. The reacted bands were visualized using Enhanced Chemiluminescence (ECL) (GE Healthcare, Waukesha, WI, USA) and ImageQuant LAS 4000 (GE Healthcare, Waukesha, WI, USA). ImageJ software was also used to analyze the reacted spots.

The IgE reactivities of the different recombinant proteins were also evaluated by ELISA assays, according to a protocol previously developed by our group [4]. Briefly, high binding plates (Greiner Bio-One MICROLON™) were coated with 5.0 $\mu\text{g.mL}^{-1}$ of recombinant Der p 21 wt or the mutant variants, in a carbonate-bicarbonate buffer (pH 9.6–9.8), for 12 h at 4 °C. The plates were also blocked using a 10% BSA and 0.05% tween-20 PBS solution. The plate wells containing each Der p 21 variant was incubated with the positive or negative serum pool (diluted 1:5 in the blocking solution) for 16 h at 4 °C. The plates were then incubated with biotinylated anti-human IgE antibody (1:2000, BD Biosciences, San Jose, CA) for 1 h at room temperature, followed by Streptavidin-HRP (1:1000, BD Biosciences, San Jose, CA) for 1 h at room temperature. Finally, 0.1 mg.mL⁻¹ TMB and 30% hydrogen peroxide were added to the plates for the reaction development. The optical density at 450 nm was measured by using a plate reader (Multiskan™ FC Microplate Photometer, Thermo Fisher Scientific, Vantaa, Finland).

For these ELISA assays, firstly, sera from 30 sensitized donors were

used to access their individual IgE reactivities against a crude extract of *Dermatophagoides pteronyssinus* or rDer p 21. Following the confirmation of the donors with the highest reactivities to rDer p 21, the sera were again used to evaluate the IgE reactivities of the recombinant mutant variants.

2.6. Statistical analysis

GraphPad Prism software was used to plot the IgE reactivity data and to perform statistical analysis. Normality was verified with Shapiro–Wilk normality test and groups were analyzed by Wilcoxon test signed rank test. A value of $p < 0.05$ was considered as statistically significant.

3. Results and discussion

Immunotherapy for HDM allergy using hypoallergenic allergens is currently considered as a promising alternative for more effective AIT [42,43]. To achieve this, recent studies have described protein engineering strategies to produce hypoallergenic mutant derivatives with reduced IgE binding, which retain epitopes recognized by T cells and induce blocking IgG antibodies [19–21,42,44,45]. The overall aim of this strategy is to overcome the negative effects of allergen-specific immunotherapy, by maintaining the immunogenicity of AIT without increasing its allergenicity, then improving the risk-benefit ratio [46,47].

In this study, we selected the Der p 21 allergen of *D. pteronyssinus* for hypoallergenization using the *in silico* mutagenesis approach, due to its high frequency of IgE recognition and allergenic activity [10,11]. Previous studies have already shown that *in silico* designed hypoallergens can exhibit a significant reduction in IgE binding, while retaining T-cell stimulating properties [42,46,48,49]. In addition to producing safer and more effective recombinant proteins for allergen immunotherapy, the *in silico* rational design of hypoallergens can also contribute to a reduction in the costs for obtaining the final product, given the reduction in experimental steps if compared to more traditional protein engineering methods such as gene shuffling [34,48,50,51].

Firstly, we performed tridimensional structure modeling, refinement, and validation of the Der p 21 wild-type allergen (Fig. 1a; Supplementary Fig. S1). Following predictions of linear and discontinuous B-cell epitopes (Supplementary Material Table S2 and Fig. S3), four amino acid residues were selected to undergo substitutions aimed at destabilization of the epitope regions of Der p 21, namely Glu(E)77, Asp (D)82, Glu(E)87, and Lys(K)110 (Fig. 1a–b). Three of these amino acid residues are present at the predicted conformational epitope E3 (Supplementary Fig. S4; Supplementary Table S2). Importantly, this region also co-localizes with an experimentally validated major conformational IgE-epitope of the Der p 21 allergen, termed peptide region P4 in the study by Curin and collaborators [44]. The computational screening for destabilizing mutations that affect B-cell epitopes was firstly used with success by Thalhamer and collaborators to generate hypoallergenic variants of the major pollen allergens of birch, Bet v 1, and timothy grass, Phl p 5 [42]. In our study, we have used the MAESTRO software [34] for the initial screening of the most destabilizing amino acid substitutions for each position, and then potential combinations of mutations were also evaluated with the aid of the FoldIt Standalone program [26]. Basically, the MAESTRO tool uses machine learning to predict changes in the Gibbs free energy ($\Delta\Delta G$) of a given protein caused by single or combined amino acid substitutions. Additionally, it is possible to scan for the most stabilizing or destabilizing mutations. Positive values of $\Delta\Delta G$ indicate destabilization and MAESTRO also provides a confidence estimation for $\Delta\Delta G$ predictions [34].

The substitution of an aspartic acid residue at position 82 of the wild-type Der p 21 protein by a proline was selected by the MAESTRO software and then ranked by FoldIt as the most destabilizing mutation (Table 1); other changes that were also ranked as the top destabilizing substitutions were: change of a lysine at position 110 by a glycine;

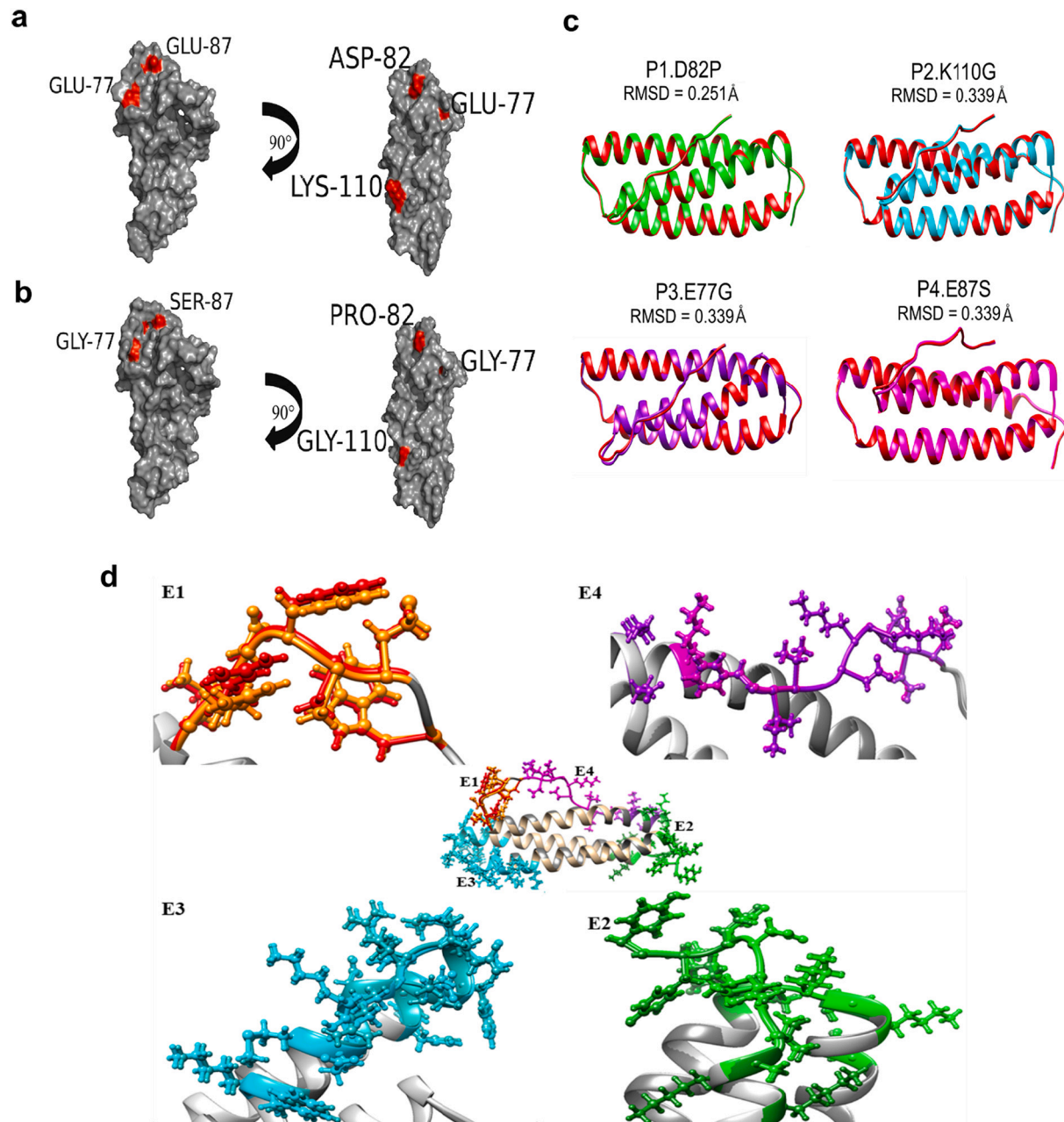


Fig. 1. *In silico* mutagenesis of the Der p 21 allergen. (a) The four amino acid positions selected for mutagenesis. (b) Amino acid substitutions predicted as the most destabilizing in the conformational epitope regions. (c) Superimpositions of the 3D models of the four mutant variants with the Der p 21 wild-type allergen. Root-mean squared deviation (RMSD) values are indicated. (d) The four IgE-epitope regions (E1-E4) identified with high confidence in Der p 21. The zoomed in regions show superimpositions of the mutant variant containing the four substitutions (P4.E87S) with the Der p 21(wt).

change of glutamic acid at position 77 by a glycine; and change of glutamic acid at position 87 by a serine (Table 1). These substitutions were combined to generate the four mutant derivatives of Der p 21: P1.D82P, P2.K110G, P3.E77G, and P4.E87S (Fig. 1a-b; Table 1). Importantly, the selected mutations do not significantly alter the three helical bundle structure of the native Der p 21 allergen (Fig. 1c-d). A similar structure has been experimentally demonstrated for homologous proteins of the groups 21 and 5 of allergens [52]. Interestingly, either drastic or non-drastic structural changes have been linked with the hypoallergenicity of a derivative for AIT [21,45,53–55]. In the case of some hypoallergenic hybrid to treat allergy caused by HDM, there was no drastic changes in comparison with the wild-type allergen [21,55].

In addition to evaluating the effect of the proposed substitutions on the structure of the epitope regions using the folded protein structures,

we employed a molecular dynamics simulation (MDS) strategy to study the potential of destabilization of these mutations on the overall structure of the Der p 21 allergen in a dynamic environment. MDS is a tool that has been commonly adopted in computational protein engineering in recent years to predict the effects of newly introduced mutations on the structural stability of engineered proteins [56–58]. It is often employed with the aim of improving the stability of mutant derivatives of biotechnologically important proteins [56,58,59]. In our study, this strategy contributed to the detection of a significant structural instability introduced by substitution of the glutamic acid at position 77 of Der p 21 (Fig. 2). Results in Fig. 2a present dynamic simulations of 100 ns duration for each of the four mutant derivatives, in addition to the wild-type allergen. The backbone root mean squared deviation (RMSD) of the Der p 21 (wt) protein showed a rapid increase in the first 10 ns of

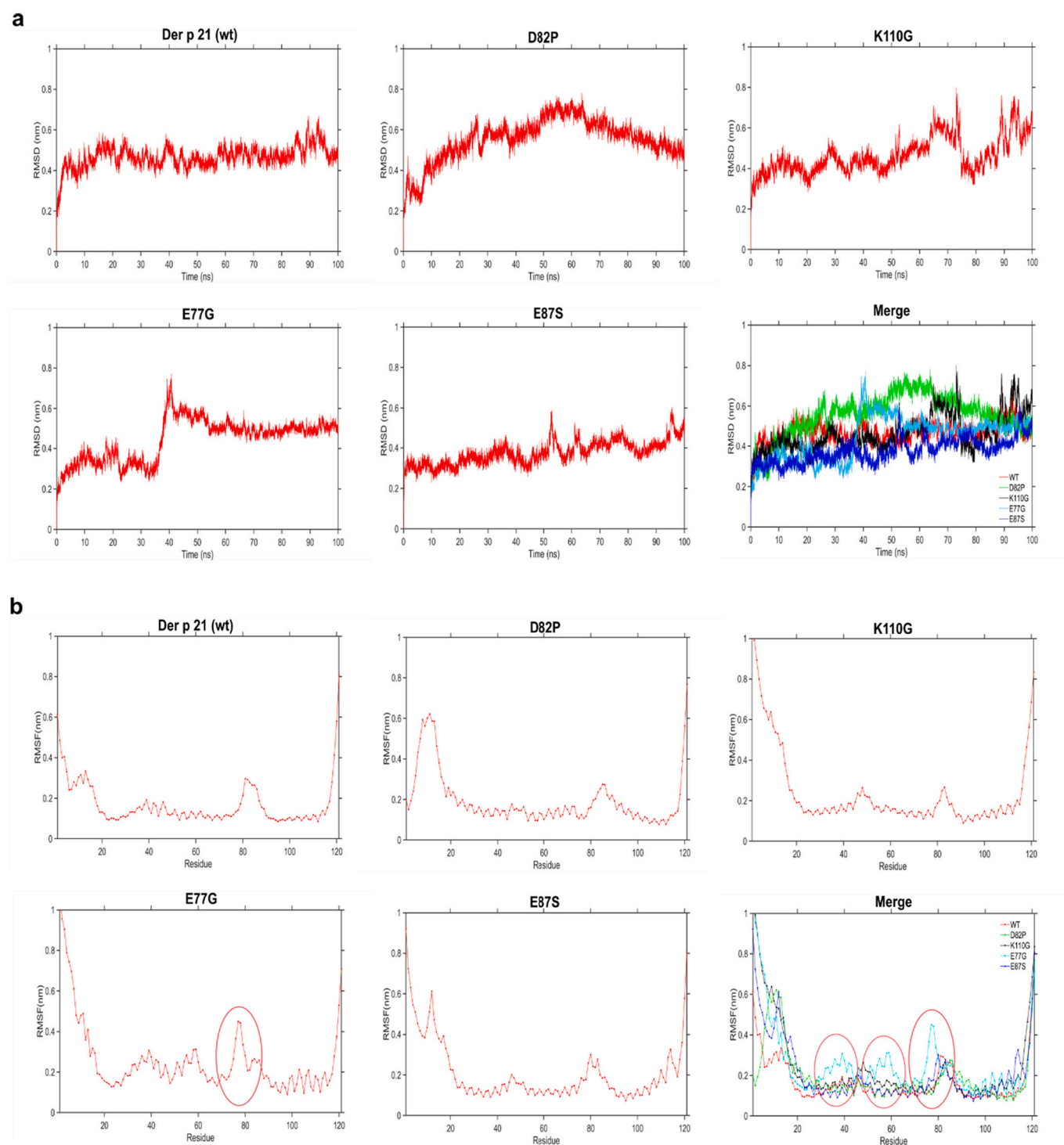


Fig. 2. Molecular dynamics simulation of the Der p 21 allergen and the four mutant variants. (a) RMSD values over a 100 ns simulation. (b) RMSF values per residue.

the simulation and rapidly stabilized at around 4 Å (0.4 nm), with a gradual increase to ca. 6 Å over the course of the experiment. The RMSD of the mutant derivatives, in turn, showed large deviations from structural stability over the course of dynamic simulation (Fig. 2a), even though all proteins had been classified as generally stable according to the instability indexes predicted by physicochemical parameters computed with the ProtParam tool (data not shown). It is noteworthy that allergens are generally considered as highly structured proteins, when compared to non-allergenic proteins, and smaller RMSD fluctuations are expected in regions that correlate with major IgE epitopes [60].

Our Root mean square fluctuations (RMSF) per residue results (Fig. 2b) clearly demonstrate that the specific amino acid substitutions performed in this study in the Der p 21 protein were responsible by large fluctuations in the epitope regions, with a significantly higher destabilizing effect attributed to the E77G mutation when introduced in the mutant protein 3 (P3.E77G) (Fig. 2b, merged panel). Interestingly, introduction of the fourth mutation (E87S) exerted a stabilizing effect on the mutant protein (Fig. 2a and b).

As aforementioned, an essential feature of a hypoallergenic protein, in order to be successfully used in next-generation allergy vaccines, is

the retention of a helper T-cell reactivity comparable to that induced by the wild type allergen, despite the reduced IgE binding ability. In our study, a prediction of MHC class II bound T-cell epitopes of the Der p 21 wt allergen and the four mutant derivatives showed a conservation on cleavage sites following the four amino acid substitutions (Supplementary Table S3). Moreover, we used the C-ImmSim server to perform a dynamic immune simulation of the potential response induced by a prime-boost vaccination scheme (days 0 and 7) with the wild-type Der p 21 allergen and the four mutant variants (Fig. 3). Fig. 3a and b shows the predicted profiles of B-cells and T-helper cells induced by vaccination with the wild-type Der p 21 allergen, over a course of nearly a year. There is a predominantly induction of a T helper 1 (Th 1) profile of cells (Fig. 3; inset chart). Notably, a two-vaccinations regimen with the wild-type Der p 21 allergen is expected to induce high levels of INF-gamma, IL-12, TGF-beta, and IL-10 (Fig. 3c). No major alterations are predicted in the productions of cytokines and interleukins, when comparing the profiles induced by vaccination with the wild-type and the four mutant variants (Fig. 3d).

To evaluate whether the Der p 21 mutant variants designed by *in silico* mutagenesis would in fact present reduced IgE-binding activity when incubated with sera of allergic patients, we firstly implemented an effort to optimize the production and purification of the recombinant forms of these proteins expressed in bacteria. Recombinant Der p 21 (rDer p 21) had already been produced and recovered in its soluble form, without fusion to solubility or purification tags, by previous studies

using *Escherichia coli* and the yeast *Pichia pastoris* as expression hosts [10,11]. In this study, we chose to introduce a 6 $\times$ -His purification tag at the C-terminus of the five recombinant proteins, through translational fusion following scarless in-frame cloning at the pOPT2.0 expression vector (Table 2; Fig. 4a). Fig. 4b shows a schematic representation of the expected rDer p 21 protein (15,846 Da) expressed from pOPT2.0, following induction by IPTG. The amino acid substitutions in the recombinant mutant variants are highlighted in the figure, and all have been predicted as soluble by analyses with the tool Protein-Sol [61,62], with solubility indexes higher than 0.6. The solubility of recombinant proteins in physiological buffers is critical for efficient and reliable production of therapeutic biomolecules, in order to overcome a well-known bottleneck in the biotechnology industry [63,64].

In fact, the successful recombinant *E. coli* clones expressed rDer p 21 and three of the mutant variants (P1.D82P, P2.K110G, and P4.E87S) in the soluble and insoluble fractions, as confirmed by 12% SDS-PAGE (Fig. 4c). These four recombinant proteins were purified to completion by affinity chromatography (Fig. 4d) with final protein yields ranging from 55.6 mg / L of culture to 60.0 mg / L of culture (Supplementary Table S4). Notably, the mutant protein 3 (P3.E77G), which was predicted as the most unstable by the dynamics simulation analysis, could not be successfully purified when expressed in *E. coli*, even when key expression parameters were modified, as suggested elsewhere [65,66] (Supplementary Fig. S5). Corroborating previous studies that also produced rDer p 21 [10,11] or rDer f 21 [52], the ortholog protein from

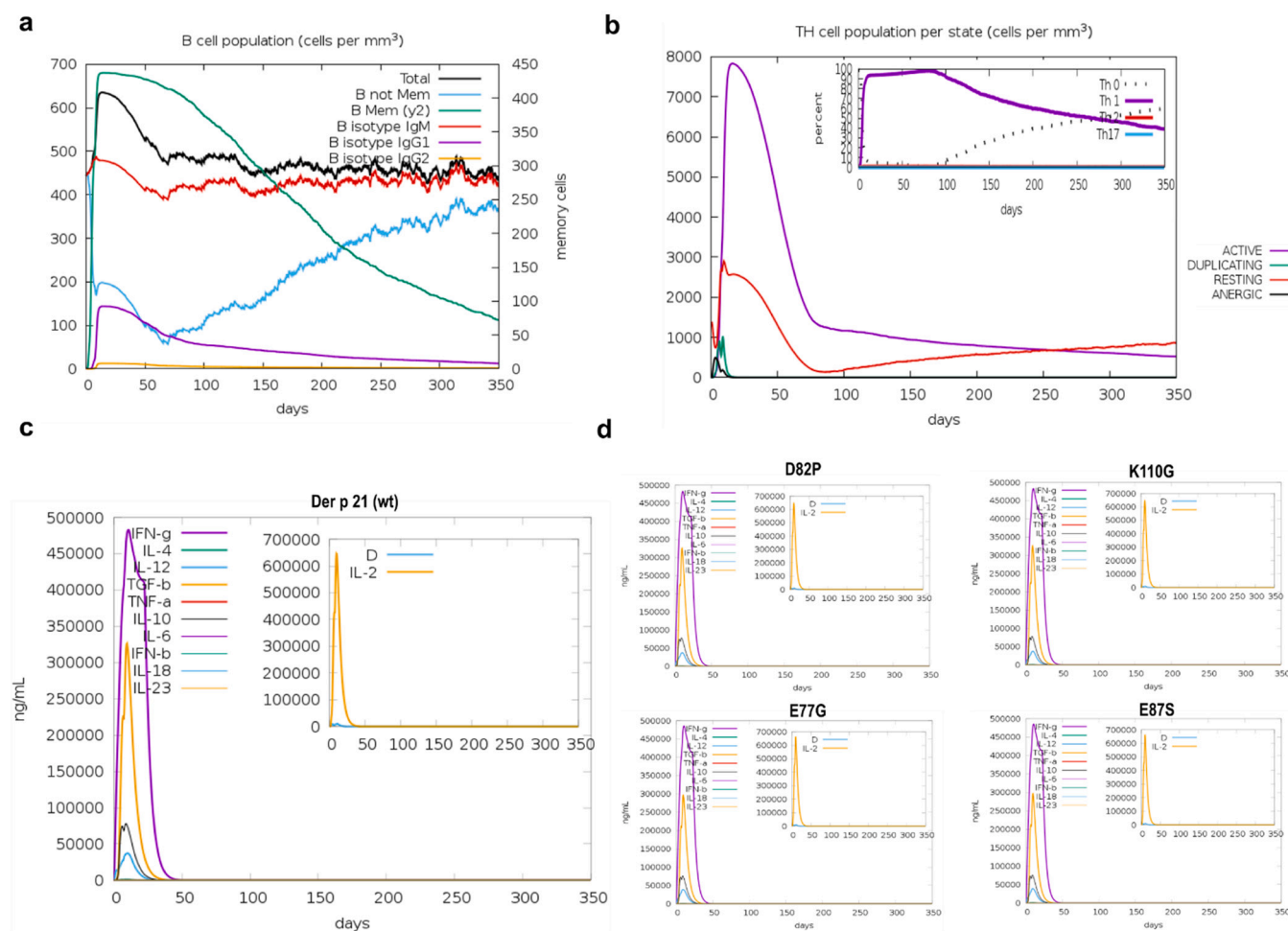


Fig. 3. Simulation of the immune response induced by vaccination with Der p 21 and the four mutant variants. (a) and (b) The profiles of B-cells and T-helper cells induced following a prime-boost vaccination regimen with the wild-type Der p 21 protein at days 0 and 7. The inset chart in (b) indicates the pattern of cellular immune response induced by vaccination. (c) Predicted cytokines and interleukins induced by vaccination with Der p 21(wt) over time. (d) Predicted cytokines and interleukins induced by vaccination with the mutant variants.

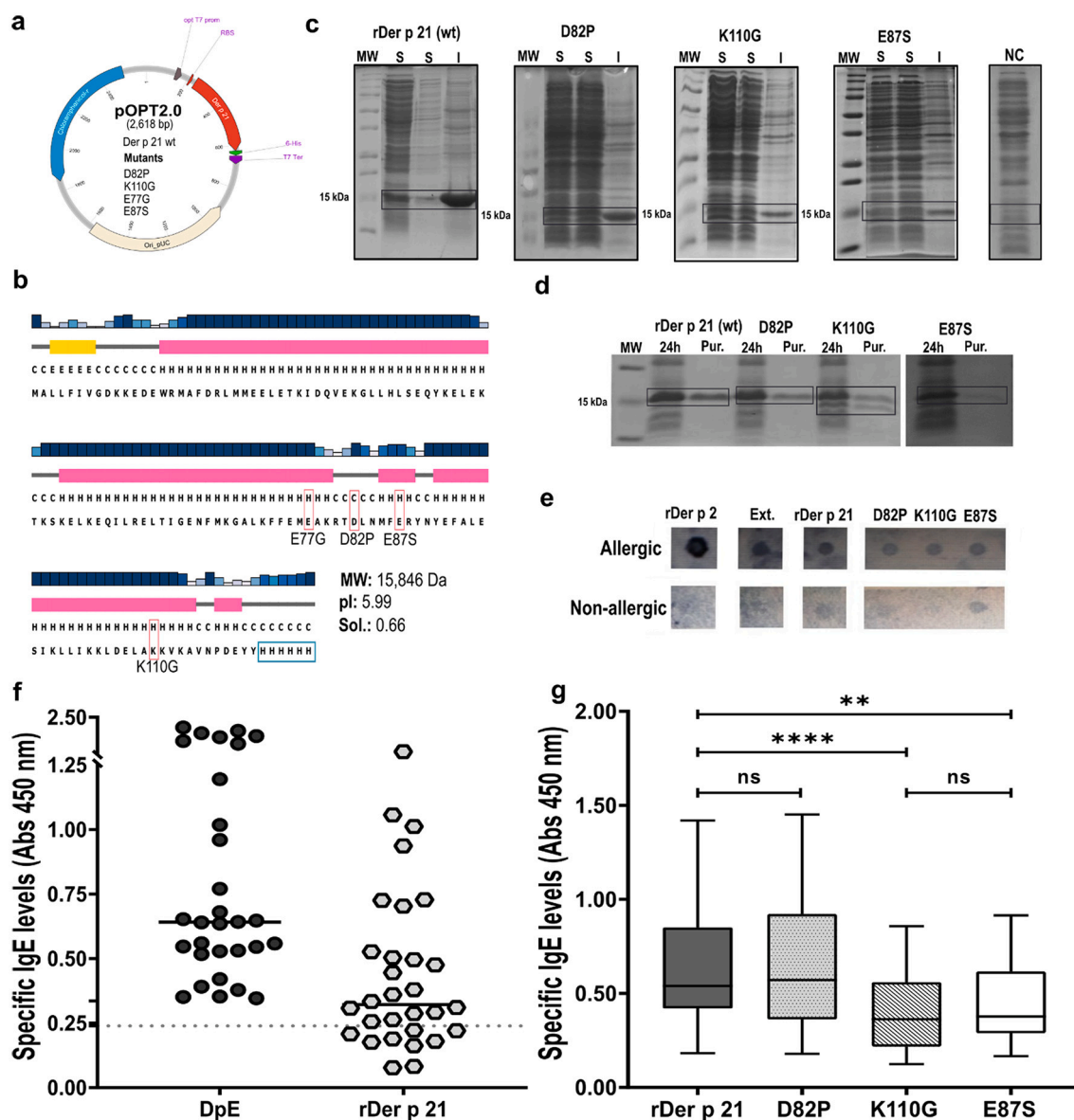


Fig. 4. Recombinant expression of the Der p 21 mutant variants and evaluation of IgE binding ability. (a) Schematic of the cloning of the gene fragments coding for rDer p 21 and the mutant variants in the pOPT2.0 expression vector. (b) Primary and predicted secondary structure of the recombinant proteins expressed in *E. coli*. The included 6x-His tag is indicated at the C-terminus. The pink regions indicate helical structures predicted by PSIPRED server. Darker blue bars indicate the confidence of secondary structure prediction per region. Molecular weight and isoelectric point of the rDer p 21 (wt) were calculated by ProtParam. Solubility score was calculated by Protein-Sol server. (c) 12% SDS-PAGE showing the expression profiles of rDer p 21 and the mutant variants. S = soluble fraction; I = insoluble fraction. NC = negative control, non-transformed BL21(DE3). (d) Purification of the recombinant proteins by affinity chromatography, following 24 h induction. (e) Dot blot analysis with sera from 5 atopic individuals. (f) IgE reactivities of atopic sera ($n = 30$) against mite crude extract (DpE) or rDer p 21. (g) IgE reactivities of rDer p 21 and the mutant variants against highly reactive atopic sera ($n = 20$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Dermatophagoides farinae, our Western blot results using an anti-His monoclonal antibody also suggest that the recombinant proteins may be found in solution partially as dimeric forms (Supplementary Fig. S6). Dimerization and aggregation can sometimes interfere in IgE-binding and hide epitopes in the allergen, leading to possible untrustworthy sensitization rates and usually reduction in reaction [67,68]. However, through dot blot analysis, we demonstrated that the wild-type rDer p 21 and all the three produced mutant variants are recognized by sera from allergic individuals ($n = 5$) (Fig. 4e), but react with different intensities. To quantify the differential levels of IgE binding, we then performed antigen-specific ELISA assays with these proteins, evaluating a set of sera from 30 *Dermatophagoides pteronyssinus*-sensitized patients. As shown in Fig. 4f, all donors reacted to the crude mite extract above the

cut-off (0.3362), but only 20 of those reacted to rDer p 21. This IgE reactivity of 66.67% was higher than previous studies in both tropical and temperate climate regions of the globe [10,11] and classifies the allergen as a major allergen in Brazilian sensitized patients. Even though the allergen was partially dimerized in solution and considering the strong reaction in half of the rDer p 21-reactive individuals (Fig. 4f), this finding may lead us to believe this allergen may be included in the allergy diagnosis in the future, especially molecular based-diagnostic tools, an important trend in the field [69,70]. Moreover, although previous studies did find limited cross-reactivity between Der p 21 and Der p 5 [44], studies on this topic for rBlo t 21 and rBlo t 5 cross-reaction with Der p 21 are still warranted. Considering the high inhibition behavior of Der f 21 against Blo t 5 and Blo t 21 IgE binding in another

study [71], cross-reactivity between these two major *Blomia tropicalis* allergens and Der p 21 is a possibility that could directly interfere in allergy diagnosis.

The twenty reactive sera of allergic patients were again evaluated for rDer p 21 IgE reaction, but in comparison with IgE reaction against the mutant variants D82P, K110G, and E87S (Fig. 4g). There is a significant reduction in the IgE reactivities of the mutant proteins K110G and E87S, whereas the variant D82P, containing a single amino acid substitution, does not significantly differ from the wild-type recombinant allergen rDer p 21 (Fig. 4g). The reactivities of the proteins K110G and E87S are not significantly different, highlighting that they are similarly hypoallergenic. The low IgE binding is a key feature in the production of an effective and safe hypoallergenic protein, suitable to be employed in AIT [19,42,72–77]. The fact that the proteins containing combinations of amino acid substitutions (K110G and E87S) presented reduced IgE reactivities when compared to the single substitution in D82P corroborates similar studies with the Blo t 5 allergen of *Blomia tropicalis*, in which it was observed that the reduction in IgE binding was more significant in variants that contained multiple mutations [78]. In addition, our group has recently shown that mutation of four amino acid residues in a hybrid protein led to a hypoallergenic derivative with low IgE reactivity in sera from both allergic non-asthmatic and allergic asthmatic donors, including low avidity features and lack of basophils' activation [21]. Noteworthy, in the present study the inclusion of the fourth mutation E87S contributed to stabilize the overall structure of the recombinant protein, without changing the profiles of low IgE reactivity and the predicted T-cell activation; this might represent an advantage for further exploration of this mutant variant to compose a potential hypoallergenic vaccine formulation for house dust mite allergies.

Although further studies are still warranted, the present study successfully translated a rational *in silico* mutagenesis design into low IgE-binding mutant variants of the allergen rDer p 21. Additionally, we obtained high yields of recombinant expression and demonstrated, for the first time, that Der p 21 can be considered a major allergen in a tropical city of Brazil.

Declaration of Competing Interest

S.P.O.S., A.B.P.L., F.S.R.S., E.S.S., C.S.P., N.M.A.N., and L.G.C.P. have filled a patent at Federal University of Bahia containing partial results of this project. It is currently pending at the National Institute of Industrial Property (INPI) of Brazil. The authors declare no other competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbagen.2022.130096>.

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