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Removal of N-Terminal Peptide Impacts Structural Aspects of an IgE-Reactive Recombinant Der p 5

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Abstract: (1) Background: Modification of the structural elements of allergens is widely used in the field of allergies. The goal of the present research was to express, purify, and characterize the shortened recombinant group 5 allergen of *Dermatophagoides pteronyssinus* (rDer p 5). (2) Methods: rDer p 5 storage stability and aggregation capacity were explored through in silico analysis, dynamic light scattering (DLS), and SDS-PAGE. Serum IgE reactivity and cytokine amount were investigated in sera or cell culture supernatants through ELISA, MULTIPLEX®, and Western blot analysis using sera from sensitized humans from Brazil, Colombia, and Ecuador. (3) Results: Dimeric rDer p 5 was detected through native PAGE, and this result was confirmed by data from DLS. The protein was thermically stable, as it did not degrade at 4 °C for 21 days. The shortened rDer p 5 was classified as a major IgE allergen in Brazil and Colombia, but minor in Ecuador. IL-13, IL-10, IL-1β, and IL-6 were significantly elevated in the sera of rDer p 5-reactive patients. The same cytokines plus IL-5 were more secreted by human cells upon rDer p 5 stimulation. (4) Conclusions: N-terminal peptide deletion led to a higher rDer p 5 folding stability, which, even though dimeric, was an IgE-reactive protein. Therefore, rDer p 5 could be used for molecular diagnostic applications or as backbone for hypoallergen design.

Keywords: allergy; *Dermatophagoides pteronyssinus*; house dust mite; IgE reactivity; recombinant allergen; thermal stability

1. Introduction

Allergic diseases have been a public health issue for decades. Worldwide, the number of humans suffering allergic asthma and/or rhinitis has progressively grown since the 1950s, when they were known as ailments typical of "first world countries" [1–3]. However, as a result of urbanization, hygiene practices, and more disseminated usage of antibiotics

and antiparasitic drugs, there has been a shift in the epidemiologic profile of such diseases in developing countries [4,5].

These illnesses can be mediated by mechanisms triggered by immunoglobulin E (IgE) and allergens. House dust mites (HDM) are known as the main source of environmental allergens responsible for several allergic sicknesses [6–8]. Among HDM, *Dermatophagoides* sp. are key triggers, and their allergenic proteins possess different sizes and functions, which are distributed in several biochemical groups [9]. Groups 1, 2, and 23 are considered the main promoters of type 2 immune responses, hence their higher frequency of IgE reactivity in humans [6,9–13].

New mite allergen groups are constantly being identified worldwide [9,11,14,15]. So far, there are 33 different groups acknowledged for *Dermatophagoides pteronyssinus* allergens [9,11]. Surprisingly, not all of them have been fully characterized globally, especially regarding IgE reactivity and some structural specificities [9,16,17]. One of those is Der p 5, an allergen mostly considered mid-tier in the aspect of IgE reactivity, specifically in Central Europe, due to percentage values mostly ranging from 30 to 50% [9,10,18]. Additionally, it has been noticed that closer to the poles this IgE reactivity decreases, whilst there was an increase in regions close to the equator [16,17,19,20], reflecting the higher importance of these allergens in the said regions. Table 1 summarizes the IgE reactivity of Der p 5 in different regions of the globe. Only two studies in countries with tropical climate have identified an IgE reaction similar to most European countries and others with temperate climates, highlighting the minor or mid-tier aspect of the allergen (Table 1).

On the other hand, this discrepancy in reactivity does not exist for *Blomia tropicalis* groups 5 and 21, which are almost always major allergens in tropical and subtropical regions of the planet [18,21–23]. In addition, both mite groups are proteins exclusive of allergenic mites [8], and they are structurally similar [23,24]. These proteins present a predominantly α-helical structure, with the presence of a flexible N-terminal region and antiparallel α-helixes that, eventually, can polymerize creating a hydrophobic cavity [25,26].

Table 1. IgE sensitization to Der p 5 around the world.

Country [1]	Frequency (%)	Reference
Australia	46.5	[27]
Austria	61.5	[28]
Austria	41.3	[29]
Belgium	12.5	[30]
China	17.7	[31]
China	17.8	[13]
Colombia	**37.8**	[32]
Germany	32.1	[33]
Germany	39.2	[12]
Italy	26.4	[34]
Singapore	**90.0**	[20]
Singapore	**11.0**	[35]
Spain	83.3	[36]
Thailand	**69.2**	[17]
United States of America	47.4	[12]

[1] In bold countries with a tropical climate; only articles published up to 2007 are listed.

Our group previously removed this N-terminal unstructured peptide from Blo t 5 [22]. The shortened rBlo t 5 did not fail to bind IgE or activate basophils in comparison with the full molecule but, in contrast, was more structurally stable [22]. This feature has made the short version of Blo t 5, not only a suitable candidate for the design of a hypoallergenic derivative, but also a better molecule for other biotechnology purposes [21,22].

Thus, our aim was to investigate the IgE reactivity against a shortened Der p 5 in tropical South American countries, evaluating whether the removal of the N-terminal

peptide would also transform the allergen into a possible candidate for personalized medicine, either for diagnosis or therapeutic applications.

2. Materials and Methods

2.1. Transformation of Escherichia coli BL21 (DE3) Star Strain

Kanamycin resistant expression plasmidial vector pET3a (ATG: biosynthetics GmbH, Merzhausen, Germany) was inserted into the aforementioned strain (Invitrogen, Carlsbad, CA, USA) using a heat-shock method. The plasmid contained the insert sequence of shortened Der p 5 (removal of N-terminus unstructured peptide), which is based in the GenBank CAA35692 sequence. This sequence is the isoform Der p 5.0101 in the World Health Organization and International Union of Immunological Societies Allergen Nomenclature Sub-committee. The shortened Der p 5 herein studied lacks only the N-terminal sequence MKFIIAFFVATLAVMTVSGEDKKHDYQNEFDFL, residue numbers 1–33. Therefore, the shortened Der p 5 is composed of the residues 34–132 of the Der p 5.0101 sequence.

Transformation was performed simultaneously with the chemical competence induction. This started with the ice incubation of an *E. coli* suspension, previously cultivated at 37 °C, for 16 h. One hundred microliters of 1M calcium chloride ($CaCl_2$) and 2 µL of the plasmid were added to the suspension. After 30 min in ice, the suspension was submitted to thermal shock in a water bath at 46 °C for 2 min, followed by another 30 min of ice incubation. Finally, there was inoculation of the bacteria suspension in 1 mL of Luria–Bertani (LB) broth medium. The inoculum was cultivated under agitation of 220 rpm, at 37 °C for 1 h. To confirm the transformation, the inoculum was plated in a Petri dish with LB agar and kanamycin, and maintained for at least 16 h at 37 °C.

2.2. Heterologous Expression of Der p 5 and Solubility Test

Heterologous expression was performed as previously described for other HDM group 5 and 21 allergens [21,22]. After transformation was confirmed, colonies were selected to begin the protein expression, initially at a small scale (5 mL of supplemented LB medium). The expression was induced by the addition of 0.5 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) when the medium optical density OD_{600} reached 0.5. Expression was conducted for 4 h, under shaking conditions of 220 rpm, at 37 °C. Heterologous expression was confirmed using 15% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and a scale-up (1 L of supplemented LB medium) was performed for the next experiments.

The whole volume of culture was centrifuged under 4000× *g*; 20 min; 4 °C. The bacterial pellet was resuspended in sodium phosphate buffer (NaP buffer), 50 mM, pH 8, at a proportion of 50 mL of buffer for each gram of pellet. The product of the resuspension was then lysed using sonication, at a frequency of 40 Hz. This method was performed in a bath ice in 8 cycles of 20 s. The lysed material was centrifuged at 13,000× *g*; 40 min; 4 °C, the supernatant collected, and the pellet resuspended in a 6M urea solution.

Samples were obtained at each step of these experiments and verified in a 15% SDS-PAGE. The presence of the protein in gel was corroborated by a band approximately of molecular weight (MW) at 12 kDa.

2.3. Immunodetection of rDer p 5 Using Western Blot Analyses

To verify the expression of rDer p 5, two Western blot analyses were performed, to immunodetect the non-tagged recombinant allergen. The first Western blot was performed following the transferring of SDS-PAGE bands from the bacterial extract to a nitrocellulose membrane (Merck Millipore, Billerica, MA, USA). Ponceau Red staining was employed to confirm the transfer. The membrane was blocked with 5% non-fat dried milk diluted in 1× phosphate buffered saline (PBS) plus 3% Tween. Then, the membrane was incubated for 1 h with serum of mice (1:2000) previously sensitized to the extract of *D. pteronyssinus*, which was obtained from an experimental model performed by the group [37]. After that, incubation was performed with a secondary anti-mouse IgG antibody conjugated with alkaline phosphatase (1:2000, Invitrogen, Carlsbad, CA, USA) for 1 h. The membrane was

exposed to a reaction solution of nitro blue tetrazolium (NBT, 0.14 mg/mL, Sigma, St. Louis, MI, USA), 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, 0.07 mg/mL, Sigma, St. Louis, MI, USA), and alkaline phosphatase buffer.

A second Western blot was performed utilizing the same samples as for the heterologous expression; however, the objective this time was the detection of human IgE against rDer p 5. Membranes were cut into strips and, following blocking, incubated with a pool of sera (1:500) of *D. pteronyssinus* allergic patients, as well as healthy ones (as negative control sera). Incubation was performed for 18 h, at 4 °C. After washing, the secondary antibody, a biotin mouse anti-human IgE (1:500), was incubated for 1 h at room temperature (RT). Strips were incubated with streptavidin conjugated to peroxidase (1:1000) for 1 h at RT. Finally, the reaction was performed with ECL, following fabricant recommendations (Thermo Scientific 32106, Waltham, MA, USA).

2.4. Purification of rDer p 5

The purification of the recombinant allergen followed three steps: anion exchange chromatography utilizing a Q HP column of 5 mL (Cytiva, Marlborough, MA, USA), the concentration of the pre-purified samples with Vivaspin® columns of 5 kDa (Cytiva, Marlborough, MA, USA), and size exclusion chromatography (Superdex TM 75 increase 10/300 GL column, Cytiva, Marlborough, MA, USA). This standardization followed the parameters previously established for the purification of non-fusion recombinant proteins [21,22]. Purity was corroborated through 15% SDS-PAGE. Total protein content was quantified by fluorimetry, using the Qubit method (Thermo Scientific 32106, Waltham, MA, USA).

2.5. In Silico and In Vitro Caracterization of rDer p 5 Structural Aspects

Considering previous reports on Der p 5 dimerization behavior [19,25,26,38], an initial in silico assessment of aggregating domains was performed using PASTA 2.0 [39]. Similarly to a previous analysis by our group [40], the amino acid sequence of shortened Der p 5 was uploaded to the PASTA 2.0 server, choosing the 100 top pairing energies and an energy threshold of 1.192 kcal/mol. For stability studies, the purified recombinant allergen was stored in NaP buffer at 4 °C (0 days to 21 days), as well as at RT (0 to 48 h). Samples were taken at these different time points for analyses with 15% SDS-PAGE. Native PAGE and dynamic light scattering (DLS) were used to assess the in vitro aggregation capacity. In the case of the latter, experiments were performed on a Zetasizer ZS90 (Malvern Instruments, Malvern, UK) at different temperatures (4 °C, RT, and 37 °C). Ten measurements were accumulated for evaluation using Zetasizer software and annotated as the mass weighted distribution of the hydrodynamic radius (R_H). Data were normalized considering the measurements of the buffer alone.

2.6. Individuals and Sera

The present study used sera of allergic and non-allergic individuals from 3 countries. The criteria for inclusion for allergy were based on clinical history, presence of symptoms at the time of blood collection, positive skin prick tests (SPT), and specific IgE (sIgE) measurements by ImmunoCAP®. An extract of *D. pteronyssinus* (DpE, Alergolatina Produtos Alergênicos Ltd., Rio de Janeiro, RJ, Brazil) was used for SPT, and diameters equal to or higher than 3 mm compared to the negative control were considered positive. In the case of ImmunoCAP® (d1, PHADIA, Uppsala, Sweden), only patients with more than 0.70 kU/L of sIgE were included as positive, with those lower than 0.35 kU/L as a negative control.

In the case of Brazil, sera of 84 individuals from Salvador, ranging from 21 to 40 years of age were included; among them, 55 were allergic and 19 non-allergic. Brazilian sera usage was approved by the Faculty of Medicine of Federal University of Bahia ethical committee (grant CAAE45376814.0.0000.5577), as well as the Ethics Committee of the Maternidade Climatério de Oliveira of Bahia/Federal University of Bahia (MCOB/UFBA), CONEP 15782—n° 25000.013834/2010-96, as part of a project "Risk factors, biomarkers and

endophenotypes of severe asthma". Specifically, this Brazilian cohort included patients attending the Program for Control of Asthma (ProAR), whose main goal is to provide comprehensive medical care to such patients [41,42]. For Colombian sera, 30 adult individuals (25 to 32 years of age) from Villavicencio were included as allergic and 10 non-allergic. The ethics committee of the General Board of Investigations from the University of Llanos, Colombia, approved the experimental procedures of blood collection and SPT, under 50.100.2.249 approval. For the sera from Ecuador, after approval (9-12-04) from the Ethics Committee from the Hospital Pedro Vicente Maldonado, the procedures already mentioned were performed in children from Esmeraldas Province. Thirty were considered allergic and nine non-allergic. Informed written consent was obtained from a parent or legal guardian. The documents were archived in the respective laboratory or responsible organization from each country.

2.7. IgE Reactivity Assays and Descriptive Analysis

Based on previously published papers by our group [37,43], an indirect ELISA was performed to determine IgE reactivity against rDer p 5. The cut-off was defined according to the mean plus two standard deviations of the absorbance results taken from all negative control serum. Raw data were analyzed and blanked results were plotted into graphs using GraphPad Prism 8.4.3 (San Diego, CA, USA, www.graphpad.com, accessed on 19 July 2023). Spearman's rank correlation coefficient was determined to verify possible correlations between ELISA results with rDer p 5 and DpE as coating antigens. p values ≤ 0.05 were considered statistically significant.

2.8. Determination of Cytokine Amount in Sera and Statistical Analyses

Cytokines were measured in the sera of 62 Brazilian asthmatic patients included in the study. Nine cytokines were quantified simultaneously using Luminex technology and a HCYTOMAG-60K Assay kit (Millipore, Darmstadt, Germany), following the manufacturer's instructions. The choice of cytokines was based on a previous study of the same asthmatic Brazilian cohort [44] and only cytokines with feasible amounts in sera were used with our multiplex kit. Mean fluorescent intensities were determined, and MILLIPLEX® Analyst 5.1 (Millipore, Darmstadt, Germany) was used for the analyses. The results were also transformed into graphs using GraphPad Prism 8.4.3 (San Diego, CA, USA, accessed on 19 July 2023). A Mann–Whitney test was used to confirmed whether the concentration of a given cytokine was significantly higher in rDer p 5 reactive sera than in those non-reactive. p values ≤ 0.05 were considered statistically significant.

2.9. Culture of Peripheral Blood Mononuclear Cells

Secreted cytokines induced by rDer p 5, DpE, and culture controls (lipopolysaccharide and phytohemagglutinin) were evaluated in peripheral blood mononuclear cells (PBMC). Ten randomly selected non-asthmatic allergic patients were included in this cell culture. PBMC were isolated from venous blood, after centrifugation using a HISTOPAQUE 1077® solution (Sigma Aldrich, St. Louis, MI, USA). Cells were incubated in 96-well plates (2×10^5 cells/well) in a humidified atmosphere of 5% CO_2 at 37 °C in supplemented RPMI 1640 medium (GIBCO, Grand Island, NY, USA). Polymyxin B was used in almost all wells, apart from positive controls. This antibiotic inhibited the effects of LPS present in samples of the recombinant allergens. The concentrations of cytokines were determined using commercially available ELISA duo-sets (Pharmingen, BD Biosciences, San Diego, CA, USA), according to manufacturer's instructions. Other information about this type of assay is detailed in several papers published by our group [21,22,37].

3. Results

3.1. Escherichia coli BL21 (DE3) Star Strain Is a Suitable rDe p 5 Expression Vector

Figure 1a shows the successful heterologous expression of the shortened rDer p 5. We verified a higher intensity band in MW around 12 kDa, which corresponded to the lanes

for 4 h of expression (Figure 1a). Under this reducing condition, this MW was identical to the theoretical, corresponding to the primary sequence of the shortened protein. It was also observed that, due to the lack of the same protein band in the fourth and fifth wells, the recombinant allergen was completely soluble.

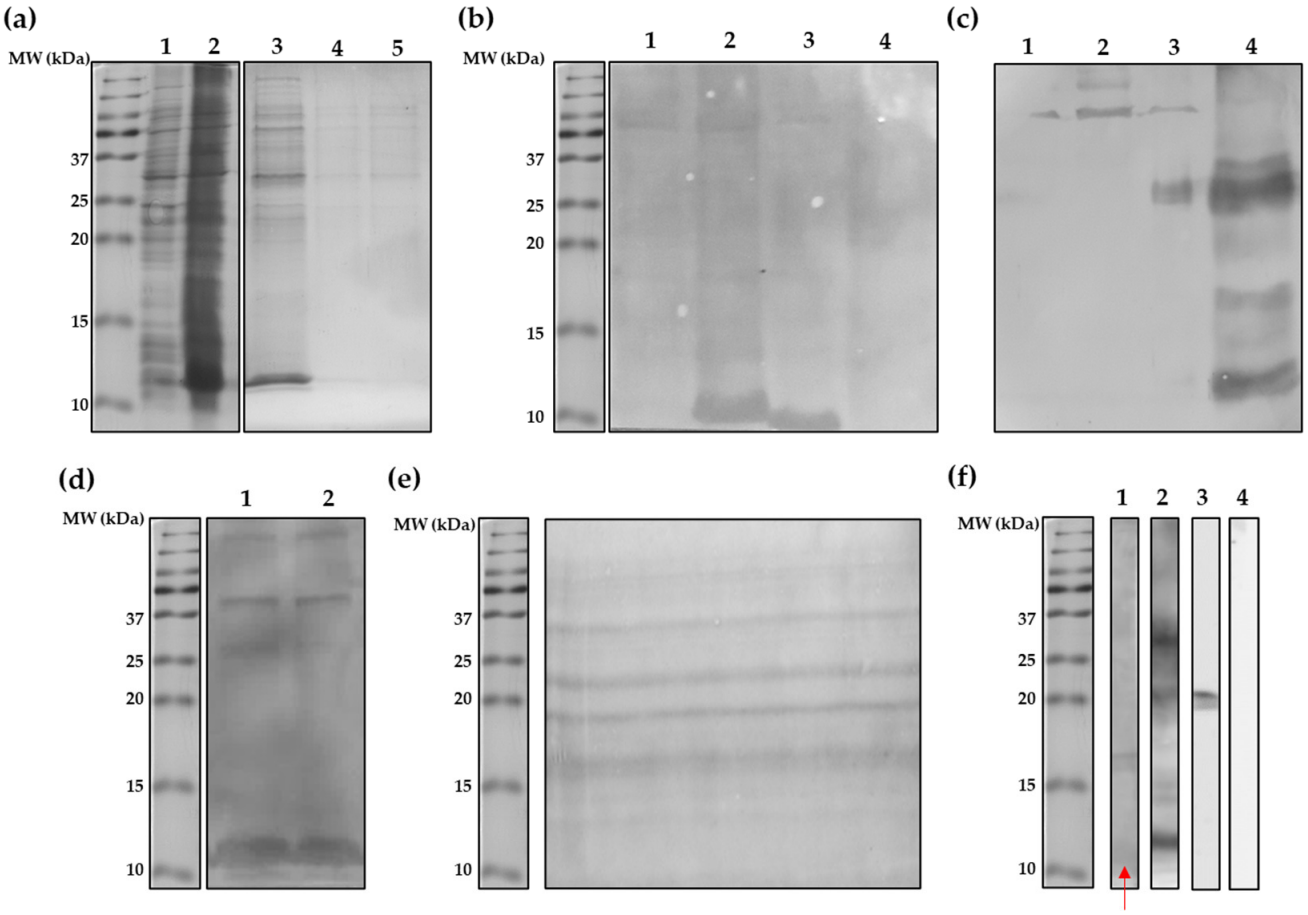

Figure 1. Expression and immunodetection of the shortened rDer p 5. (**a**) 15% SDS-PAGE for the expression and solubility test (Lane 1—0 h, before IPTG induction; Lane 2—4 h of expression, OD_{600} = 0.5; Lane 3—soluble fraction of bacterial extract; Lane 4—soluble fraction of bacterial extract in NaP buffer; Lane 5—solubilized-bacterial pellet in 6M Urea); (**b**) (Ponceau staining) and (**c**) (NBT/BCIP reaction): nitrocellulose membrane for Western blot analysis using a pool of mice sera allergic to an extract of *D. pteronyssinus* (DpE); Lane 1—0 h, before IPTG induction; Lane 2—4 h of expression, OD_{600} = 0.5; Lane 3—soluble fraction of bacterial extract; Lane 4—DpE (25 µg/well). (**d**) Ponceau staining displaying the soluble fraction of bacterial extract in lanes 1 and 2. (**e**) Ponceau staining of DpE at 125 µg/well. (**f**) NBT/BCIP reaction on strips; lanes 1 and 3—soluble fraction of bacterial extract; lanes 2 and 4—DpE; The red arrow indicates the rDer p 5-reactive band; Pool of *D. pteronyssinus* reactive sera were added in lanes 1 and 2, whereas lanes 3 and 4 added sera from controls.

Figure 1b,c reveal that there was no IgG anti-rDer p 5 in the sera of mouse, possibly indicating the absence of this protein in the DpE used for sensitization, which is a commercially available extract. This result led us to try a second Western blot analysis (Figure 1d–f) using human sera. We confirmed the reaction in corresponding band, although it was faint. DpE, in contrast, was IgE-reactive in several bands (Figure 1f).

To confirm the expression, we employed anion exchange chromatography to purify (Figure 2A,B) rDer p 5. The allergen showed low affinity to the column, since the protein was not eluted in eluted fractions, but it was only present in the flow through and during column wash. Nevertheless, with this chromatographic step, the protein was partially purified,

considering that other fractions had shown signs of bacterial proteins. However, it was necessary to concentrate the sample, and then we acquired fractions with a higher degree of purity. Figure 2C,D show the successful homogeneity and purity of the recombinant produced herein, considering the constancy of the size exclusion chromatography and lack of impurity bands in the reducing gel.

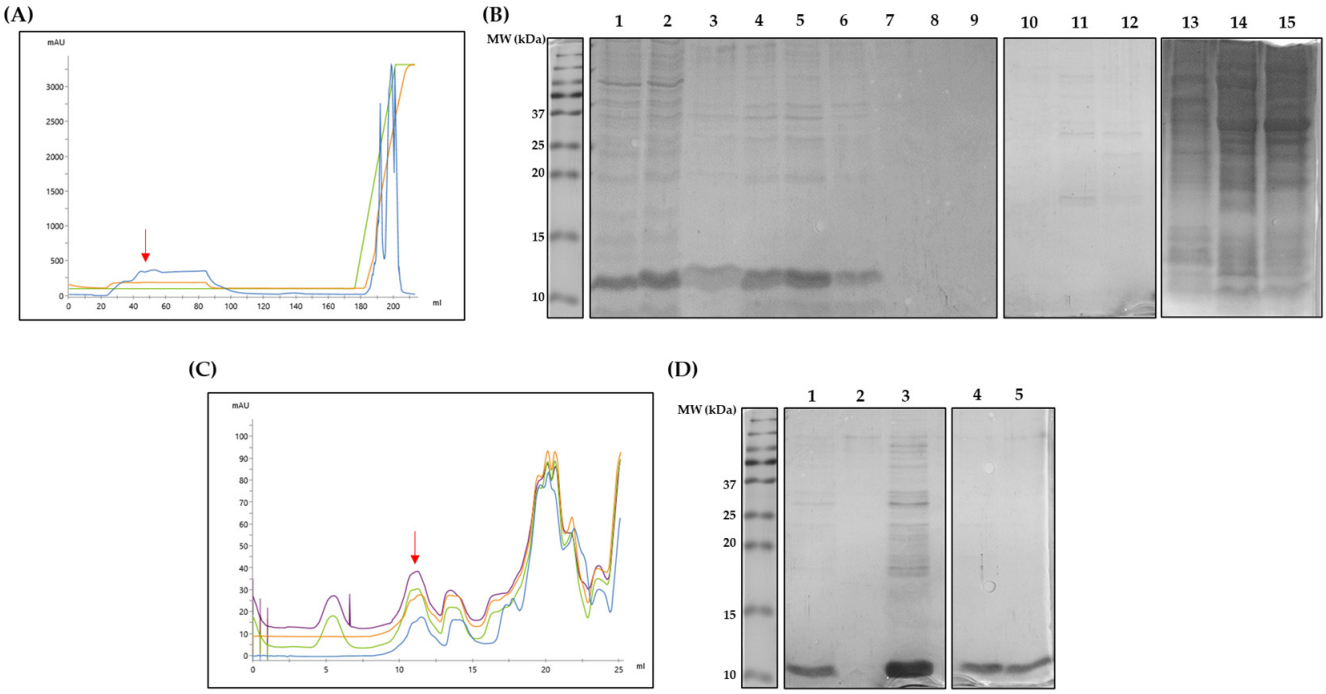

Figure 2. Purification of the shortened rDer p 5. (**A**) Chromatogram of an anion exchange purification (orange—conductivity, green—B buffer concentration, blue—mAU 280 nm). Red arrow indicates the recombinant protein chromatographic pike. (**B**) 15% SDS-PAGE for identification of purified rDer p 5 (Lane 1—flow through; Lane 2—column wash; Lane 3 to 15—eluted fractions). (**C**) Four size exclusion chromatography purifications are displayed with overlapping chromatograms. Red arrow indicates the recombinant protein chromatographic pike. (**D**) 15% SDS-PAGE for the final purification steps of rDer p 5. (Lane 1—pool of samples form anion exchange chromatography; Lane 2—filtered samples obtained after centrifugation with a Amicon® column; Lane 3—concentrated sample for purification; Lanes 4 and 5—representative purified fractions).

3.2. Shortened rDer p 5 Is Structuarally Stable but Agregates

As a way to prove the stability, the shortened rDer p 5 was stored under physiological conditions at 4 °C and at RT. Although at 4 °C there is no sign of degradation (Figure 3A), at RT the protein seemed to become shorter (Figure 3B). Nevertheless, we still believe that the protein was stable, considering the fact that, at 48 h, the protein did not degrade completely at RT (Figure 3B). However, comparing different concentrations of the purified allergen in a 15% SDS-PAGE (Figure 3C) and a native-PAGE (Figure 3D), the rDer p 5 showed signs of dimerization behavior. To further investigate the aggregation behavior in solution, DLS analysis was performed at different temperatures. As shown in Table 2, under pH 8 in a NaP buffer, the protein displayed both a monomeric and aggregation behavior. At RT, the protein seemed to only dimerize, given the R_H of 1.57 ± 1.89 nm and 15.59 ± 7.64 nm. Table 2 also shows that for the other tested temperatures, besides the monomeric form of the shortened rDer p 5, the different R_H were characteristic of oligomerized proteins in solution.

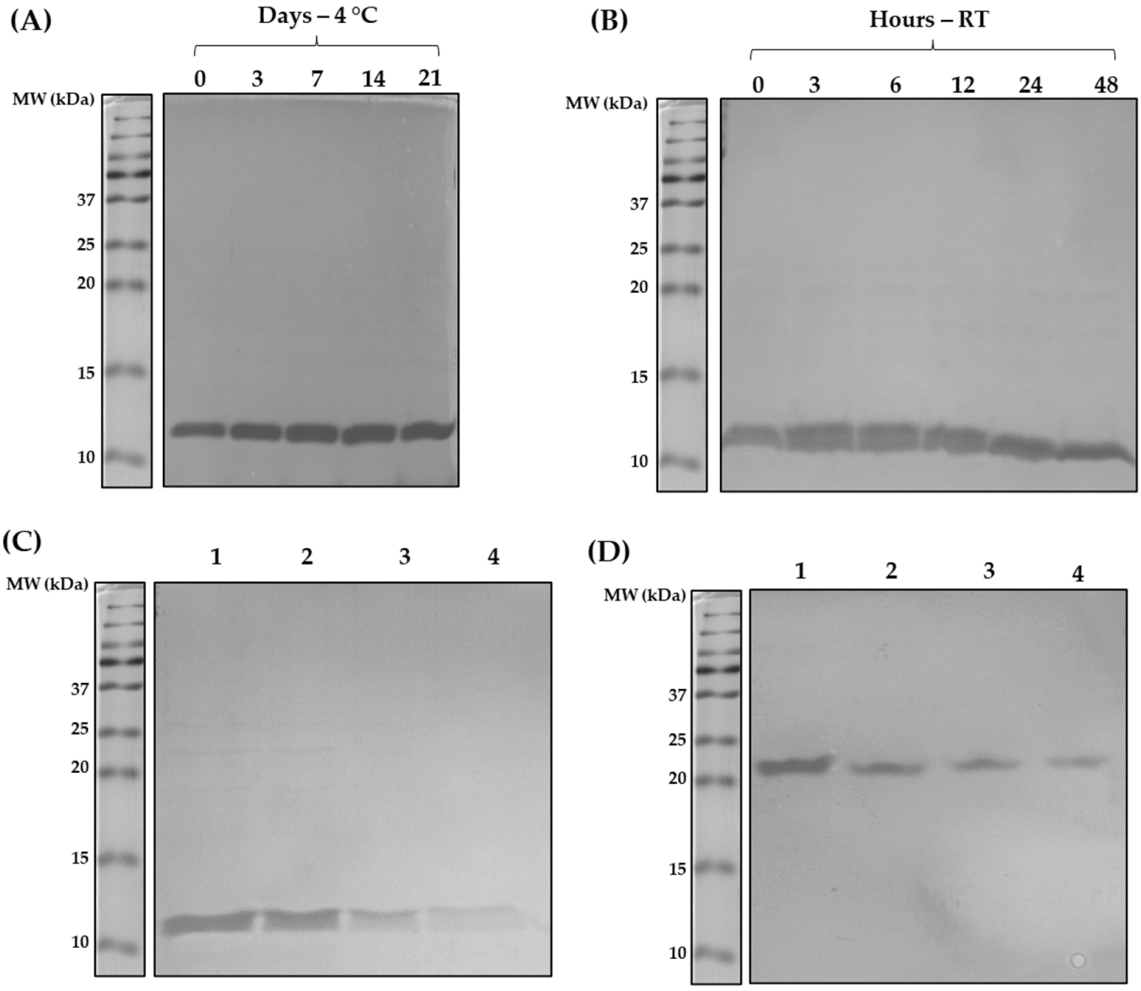

Figure 3. Structural in vitro analysis of the shortened rDer p 5. (**A**) 15% SDS-PAGE of stability of the recombinant at 4 °C, during the days displayed in the illustration. (**B**) 15% SDS-PAGE of stability of the recombinant at RT, during the periods displayed in the illustration. (**C**) 15% SDS-PAGE of purified shortened rDer p 5 at 10 (lane 1), 5 (lane 2), 2.5 (lane 3), and 1.25 (lane 3) µg/well. (**D**) 15% native-PAGE of purified shortened rDer p 5 at 10 (lane 1), 5 (lane 2), 2.5 (lane 3), and 1.25 (lane 3) µg/well.

Table 2. In solution aggregation of shortened rDer p 5.

Temperature (°C)	R_H [1] (nm)	
	Pike 01	Pike 02
4	1.130 ± 0.841	135.832 ± 122.365
25	1.573 ± 1.894	15.588 ± 7.640
37	1.490 ± 0.715	78.000 ± 67.197

[1] Hydrodynamic radius.

3.3. Shortening of rDer p 5 Does Not Change Its Aggregation Behavior

The results of in silico analysis of the primary structure of rDer p 5 are displayed in Figure 4. The most aggregation-prone sequences were, in fact, identified inside the second helix, between residues 38 and 56 (Figure 4a). Figure 4b shows the top-paring values determined using PASTA 2.0. IVAEMDTIIAMIDGVRGVL residues exhibited the highest pairing and linear probability, and the aggregation and disorder profile (Figure 4c) confirmed the results, interestingly with top values for TIIAMID and not GVRGVL. While the aggregation-prone region showed a high helix probability (Figure 4d), no β-strand probability was found during the PASTA 2.0 analysis.

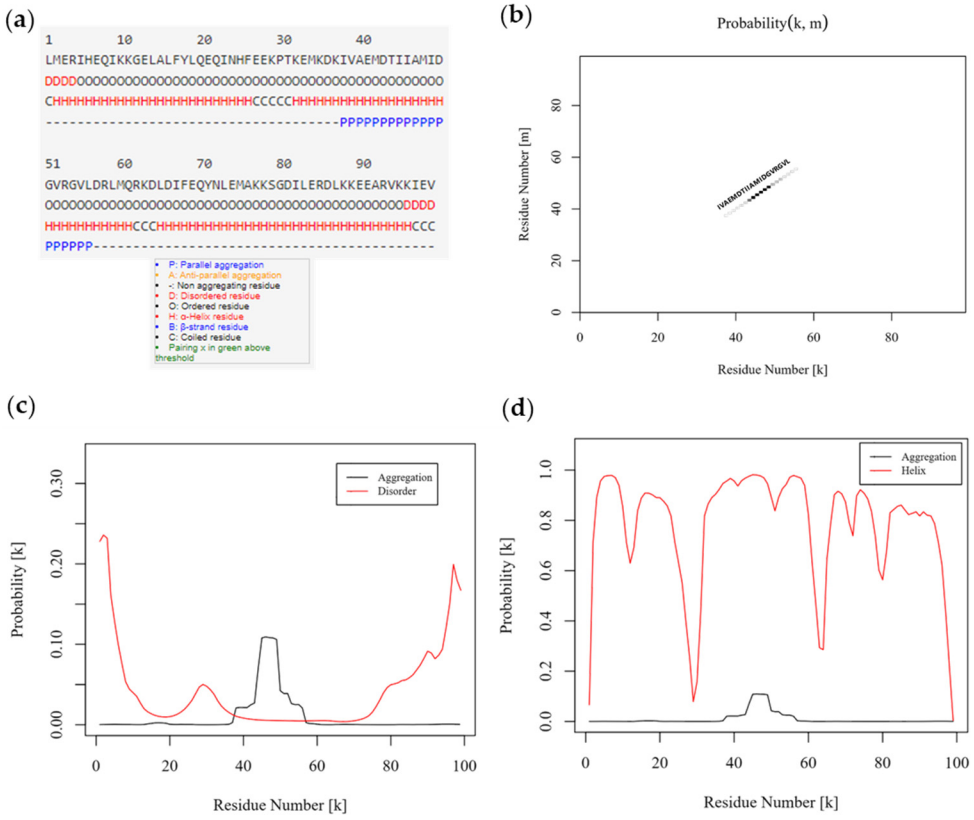

Figure 4. In silico aggregation behavior analysis. (**a**) Residue assignment of secondary elements and parallel aggregation region identified using the PASTA 2.0 server [39]. The sequence of the shortened Der p 5.0101 was used in this analysis and it lacked residues 1–33 of the original sequence. (**b**) Pairing and linear probability profiles as a function of the residue position. Key residues were added in the plot. (**c**) Aggregation and disorder profiles. (**d**) Aggregation helix profile.

3.4. Shortened rDer p 5 Is IgE-Reactive

Figure 5a shows the IgE reactivity among the triad of countries. All patients included in the study reacted to DpE above the cut-off line of 0.142. However, there were some individuals that reacted close to this line. As expected, not all patients reacted to rDer p 5, and using the traditional classification we could affirm that the shortened protein was a major allergen in Brazil and Colombia, while being a minor one in Ecuador. Specifically, the frequencies were: (i) 57.3% for Brazil (55 out of 96 allergic patients); (ii) 60.0% for Colombia (18 out of 30 allergic patients); and (iii) 6.67% (2 out of 30 allergic patients). Ecuador's population showed a slightly higher DpE median compared to the other countries (Figure 5a). Moreover, when we correlated DpE and rDer p 5 IgE reactivity, there was a significant positive correlation between the antigens (Figure 5b).

3.5. Pro-Inflammatory Cytokines Were More Present in Sera of rDer p 5-Reactive Patients

Randomly selected Brazilian sera were used for the measurement of several cytokines. While the levels of the type 2 cytokine, IL-13, were significantly higher in the sera of rDer p 5 reactive subjects compared to non-reactors, there was no difference in IL-5 levels between the two groups (Figure 6). Interestingly, IL-10 was also detected at significantly greater concentrations in rDer p 5 positive patients (Figure 6). Levels of IL-1β and IL-6 pro-inflammatory innate cytokines were also significantly elevated in the sera from rDer p 5 reactive subjects. The same figure shows trends of greater concentrations of IL-12 p70 and IL-8 in reactors, but no differences for IFN-γ and TNF-α.

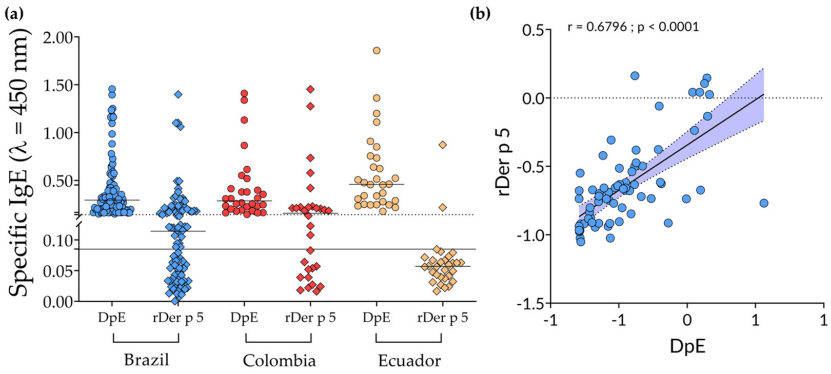

Figure 5. IgE reactivity assays. (**a**) IgE reactivity per sample group, addressing individuals with reactivity to *D. pteronyssinus* and rDer p 5. The cut-offs are represented by a horizontal dotted line for DpE assay (0.142), and a horizontal straight line for rDer p 5 assay (0.085). Different colors were used to represent each country. Blue for Brazil, red for Colombia, and yellow for Ecuador. (**b**) Spearman's correlation of IgE against rDer p 5 and DpE (n = 75) displayed in log scale. DpE—*D. pteronyssinus* extract.

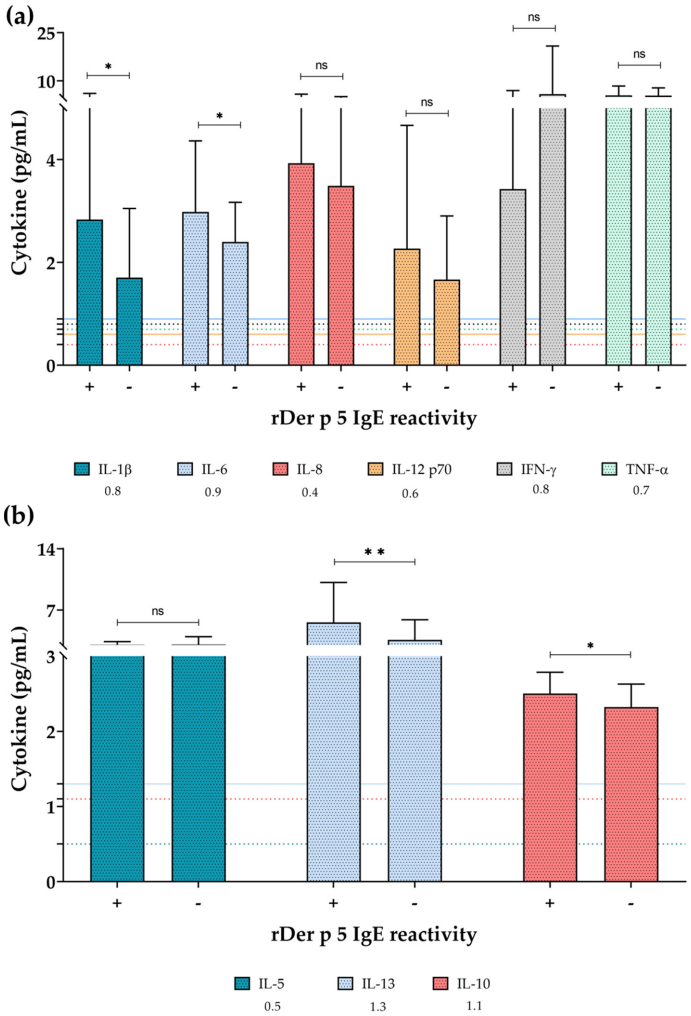

Figure 6. Cytokine amount in the sera of reactive (positive) and non-reactive (negative) rDer p 5 Brazilian patients (n = 62). (**a**) Proinflammatory cytokines. (**b**) Type 2 cytokines and IL-10. Luminex technology and HCYTOMAG-60K assay kits were used for measurements. Dashed and straight lines correspond to minimum detectable concentrations, which are written below each cytokine in the legends. Mann–Whitney test was used for comparison. * $p \leq 0.05$; **: $p < 0.01$. ns: non-significant.

3.6. rDer p 5 Induces the Secretion of Type 2 and Proinflamatory Cytokines

The cytokine profile shown in Figure 7 indicates that rDer p 5 may induce T helper cells response. rDer p 5 induced significantly higher levels of IL-1β, IL-6, IL-10, IL-5, and IL-13 when compared to unstimulated cells. Some typical type 1 cytokines were not significantly induced by the recombinant allergen. DpE stimulus behaved similarly but it induced the secretion of TNF in a significant manner in comparison with unstimulated cells.

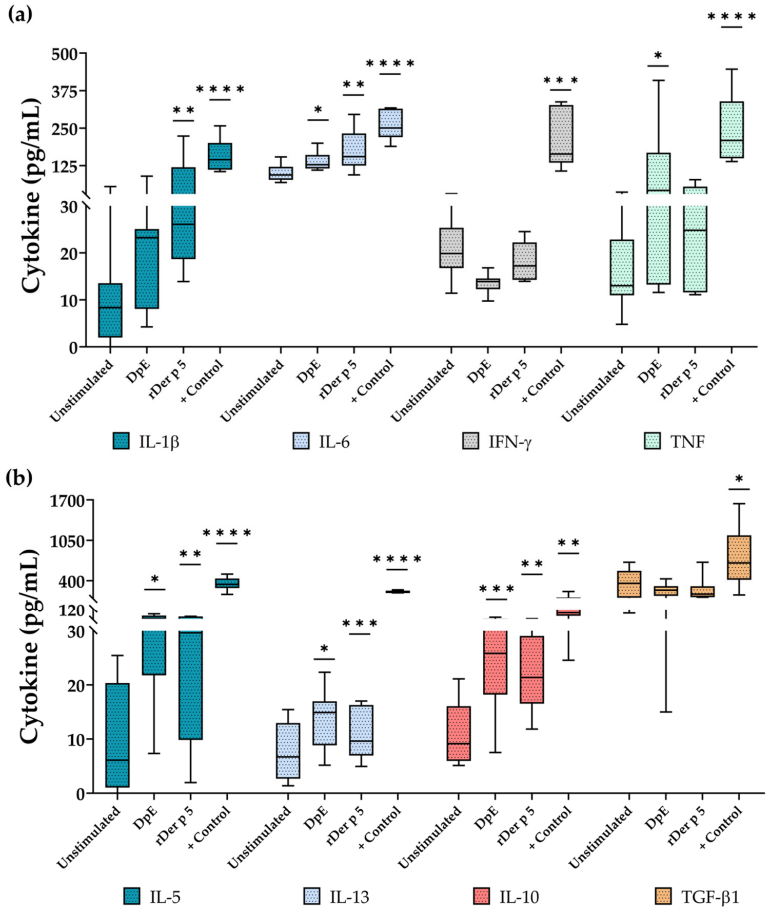

Figure 7. Cytokines in peripheral blood mononuclear cell supernatants (n = 10). The amounts of (**a**) proinflammatory cytokines and (**b**) type 2 and regulatory cytokines in PBMC cultures of *D. pteronyssinus*-allergic donors are shown in box plot. Different stimuli are displayed on the X axis. ANOVA or Friedman tests were used for statistical analysis. Dunnett or Dunn post-tests were used after the first test. * $p \leq 0.05$; **: $p < 0.01$. ***: $p < 0.001$ ***: $p < 0.0001$, when compared to unstimulated cells. DpE—*D. pteronyssinus* extract.

4. Discussion

Investigation of the structural and immunological features of allergens is essential to establish their relevance for diagnostic and therapeutic purposes. Evidently, isolation of natural allergens would better establish their precise relevance to physicians and researchers in the allergy field. However, the purification of natural allergens from their original allergen sources can be rather difficult, requiring extensive purification steps to achieve real purity [45–47]. Additionally, the number of monoclonal antibodies available for use in affinity chromatography is currently scarce, especially in the case of certain allergic sources [48,49]. For this reason, most research groups tend to heterologously express allergens and characterize them in vitro first [16,19,26]. With a few exceptions, most recombinant allergens displayed identical or similar allergenic activity and IgE binding capacity [43,45,50].

DNA recombinant technology has brought many advances in the allergy area, allowing not only the recombinant production of wild-type allergens [9,23,51], but also the possibility of specific modifications in their amino acid residue sequences [22,52]. Several articles are currently available that detail modifications to reduce IgE reactivity (hypoallergenic derivatives) or simply to increase allergen structural stability [11,53].

Our group, for instance, has shown how the removal of a N-terminal unstructured peptide led to an increase in thermal stability and resistance to endolysosomal degradation [11,21]. Although both allergens, rBlo t 5 and Blo t 21, retained IgE binding and allergenic activity, their increased thermal stability and stronger resistance towards proteolytic degradation made them more fit as backbones for the construction of hypoallergenic derivatives [11,21]. We believed, therefore, that other mites may have group 5 and 21 allergens that behave similarly.

As shown by the results detailed herein, we achieved some of our goals: the heterologous expression of a group 5 mite allergen of *D. pteronyssinus* (rDer p 5), a method to fully purify a recombinant shortened Der p 5, and a thermically stable rDer p 5, which has shown high IgE binding capacity in the patients of some countries.

Even though these feats are positive, certain issues must be discussed further. First, in reducing conditions, the allergen migrated as a monomeric protein, but in native PAGE and in solution, rDer p 5 dimerized and aggregated. This behavior can indeed influence allergenicity and, sometimes, lead to a reduction in IgE binding [40,54,55]. However, we predicted such results, considering the fact that only the N-terminal peptide was removed from the sequence. The key amino acid residues responsible for dimer formation were kept in our recombinant. Other authors [25,38,56] have shown how Der p 5 owns one of the strongest stabilizing hydrophobic surfaces, compared to other group 5 and 21 mite allergens. This feature was explained by the presence of valine residues in such regions, which create, in turn, a hydrophobic zipper domain [25,38]. According to our in silico analysis, however, other residues are prone to dimerization, especially certain isoleucine residues. Khemili et al. also identified some isoleucine residues as important for dimer formation in their study [38].

Shortening rDer p 5 may have made it thermically stronger and, in turn, a more stable allergen. This inference was strengthened by the stability assay upon extended storage, as displayed in the results section. Strong resistance towards degradation is a very appealing feature for vaccine candidates, and this strong stability at 4 °C for 21 days of assay had been observed previously [57]. Additionally, the deletion of the N-terminal region caused an increase in the thermal stability of shortened rBlo t 5 [22], which was posteriorly used as a backbone to design a hypoallergenic hybrid protein [21]. As a further note on this topic, the deletion, mutation, or truncation of N-terminal domain of proteins can increase the proteins' thermal stability, in some cases from 10 to 30 °C [58,59]. For instance, at room temperature, the shortened version of rBlo t 5 slowly lost its secondary structure elements compared to the full molecule [22]. Although we did not perform circular dichroism assays, we have confidence that the shortening of rDer p 5 might have led to higher thermal stability than *B. tropicalis* group 5 and 21 allergens, considering that our rDer p 5 did not degrade as strongly as its *B. tropicalis* counterparts after 48 h of RT exposure [21,22]. We believe that the allergen's dimerization capacity associated with the compactness of the molecule after N-terminal peptide deletion may have created a more stable rDer p 5.

Nevertheless, it is known that dimerization can also decide the fate of allergenic response in other directions, increasing responses or not showing effects at all [56,60]. Niemi and collaborators, for instance, revealed that lipocalins are less allergenic when monomeric in solution, with a lower ability to induce the release of inflammatory mediators compared to dimeric variants [61]. The allergenic properties of the birch pollen allergen, Bet v 1, is also highly dependent on dimerization. Bet v 1 dimerization provides two IgE binding sites that are required for cross-linking of IgE in effector cells and BCRs in B lymphocytes [56,60]. Interestingly, all these aforementioned dimeric allergens are known lipids carriers, as is rDer p 5 [16,19,26]. However, the investigation of IgE binding of a

monomeric rDer p 5 in comparison with our oligomerized one would still be warranted, and we still believe that our IgE frequency was a relevant result.

In this sense, it was possible to classify rDer p 5 as a major allergen in study samples from Brazil and Colombia but a minor allergen for the Ecuador samples. Despite the fact that this classification is still used, authors have stated that it is time to put aside this classification in allergology [40,62]. Minor allergens, for example, may indeed be clinically relevant and several have been linked to innate immune mechanisms, including rDer p 5 [16,63,64]. Others have been associated with mild to severe allergic symptoms [18,65–67]. Walsemann et al. revealed that rDer p 5 can be a marker of asthma and the severity of atopic dermatitis [66]. In future studies, with a more representative sample size, we intend to assess whether rDer p 5 will serve as a marker of the severity of allergic diseases in Brazil.

The IgE reaction to an allergen may vary, depending on several factors, such as the genetic background and environmental factors of a population [68,69]. Concerning environmental factors, Villavicencio, Salvador, and Esmeraldas display environmental particularities despite the fact their climates are relatively similar, which is tropical to subtropical [68,70,71]. Villavicencio is surrounded by a rainforest in the rural area of Colombia, whereas Salvador and Esmeraldas are coastal cities. It has been stated that these factors could influence the exposure to allergens. Individuals exposed to different isoforms of allergens, subject or not to certain infections, or even inhabiting regions with different climates and altitudes, will not necessarily be sensitized to the same allergens in the same amount [7,40,72,73].

One hypothesis to be raised about this low reactivity for the sera of Ecuadorian children involves the difference in age among the study populations. Studies have confirmed that the frequency of sensitization to some HDM allergens can decrease or increase with age. Ying and colleagues demonstrated how sensitization to aeroallergens gradually increases up to age 18 [74]. On the other hand, allergens such as Der p 23 seemed to have a contrary behavior in a prospective study [75]. In any case, rDer p 5 apparently behaves like a late sensitization allergen. However, it would be necessary to carry out a prospective study of these children, carrying out other screenings for IgE against rDer p 5 and thus confirm whether the sensitization of this allergen really increases with age.

It is necessary to point out that, in some previously published studies, rDer p 5 was a major allergen [17,20]. The IgE frequencies (Table 1) were even higher than ours. However, in those reports, the research groups used *Pichia pastoris* as a vector, whose disadvantage for heterologous expression is the possibility of producing hyperglycosylated proteins, which can overestimate IgE reactivity [40,76,77]. A more recent heterologous expression of rDer p 5 in yeast reported an advantage of this vector, as it allowed studying the allergen in its monomeric form, whether in solution with neutral or acidic pH [16]. Even so, considering that previous studies such as those listed in Table 1 used the same expression vector as our study, we believe that *E. coli* is still a suitable expression vector, with intrinsic advantages over eukaryotic systems [78]. For that reason, and the strong stability of our recombinant, we are sure to include this allergen in future personalized medicine tools for diagnostic purposes.

There is a trend of replacing extract-based formulations with diagnosis and allergen immunotherapy in the allergy field [45,49,53]. In fact, our results show rDer p 5 was missing in a commercially available HDM extract. At least, the so-called major *D. pteronyssinus* allergens, Der p 1, 2, and 23 were not absent from this extract [37]. The literature has already pointed to the low concentration or complete absence of Der p 5 in commercial extracts [10,79,80]. These observations confirm the need for a broader quality control of allergen extracts or their replacement with isolated molecule-based formulations. The presence of a multitude of allergens in extracts is fundamental, mainly in order to avoid misdiagnosis and to better mimic allergic diseases in experimental models; after all, humans are constantly challenged by various allergens at once [10,49,79]. However, tools such as capture ELISA kits or mass spectrometry are scarce or too expansive for some laboratories.

Concerning the cytokine results, some inflammatory interleukins were significantly more present in the sera of asthmatic patients and secreted by allergic non-asthmatic patients from Brazil with a positive reaction to rDer p 5. IL-13 is well known—besides its role on the effector phase of allergic inflammation (mucus production)—for its role in IgE production, and together with IL-4, it promotes the class-switch mechanism in plasmocytes [81,82]. Although not significantly present in sera but strongly secreted by PBMC upon rDer p 5 stimulation, IL-5 is an important cytokine for type 2 responses. The mainstream role of IL-5 is the activation and recruitment of eosinophils, one of the main effector cells in allergic inflammation [83,84]. IL-1β can be secreted by B cells, activating and strengthening their activity through participation of the plasma cells' differentiation and, in turn, triggering the production of IgE [85]. Additionally, another cytokine more present in rDer p 5-positive patients was IL-6. It is proposed that the IL-6 produced by macrophages may induce the production of classical type 2 allergic inflammation, such as eosinophil accumulation, IgE production, and mucus hypersecretion [86,87]. The higher presence of IL-10 in the sera of asthmatic patients positive to rDer p 5 was surprising but not completely unexpected. In previous studies, our group found a similar pattern of higher IL-10 production in peripheral blood mononuclear cells from allergic donors, when allergenic recombinant proteins were used as stimuli [21,37]. Although IL-10 is considered a regulatory cytokines, some studies have shown that this cytokine does not always act in this regulatory manner [88,89], and therefore the higher production of this cytokines in the sera of rDer p 5-positive patients may be indicative that further studies are required to clarify if this cytokine acts as pro-inflammatory, or if this higher production is only a consequence of steroid use. If the latter, the pharmacotherapy effect on the upregulation of IL-10 was not sufficient—as shown by other studies [90,91]—to downmodulate specific IgE production, in our case against rDer p 5.

5. Conclusions

The shortening of Der p 5 influenced its thermal stability, leading to the acquisition of a stable protein. Even though dimeric, the soluble and highly IgE reactive nature of our rDer p 5 in Brazil and Colombia indicates that the recombinant allergen can be successfully produced in *E. coli*. Therefore, the protein can be used as both a candidate for molecule-based microarrays for diagnosis, and as a backbone to design hypoallergenic derivatives for allergen immunotherapy.

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