

N-terminal peptide deletion influences immunological and structural features of Blo t 5

To the Editor,

Studies demonstrated that among the identified *B. tropicalis* allergens, Blo t 5 is a major allergen,^{1–4} which makes the protein a target for diagnosis and allergen-specific immunotherapy (AIT). However, a successful AIT requires a high safety and efficacy of the therapeutic and an extensive physicochemical and immunological characterization for candidate molecules.⁵

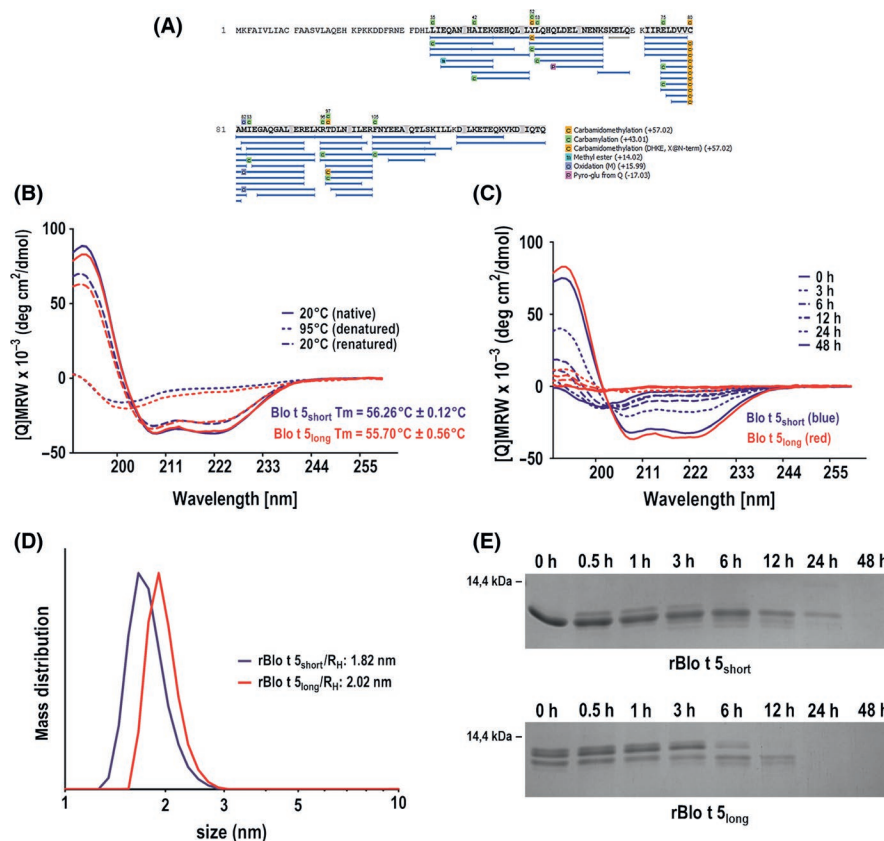
Our proteomic analysis of *B. tropicalis* extract (Figure 1A and Table S1) has revealed the absence of the intrinsically disordered N-terminal motif of nBlo t 5, composed of 18 amino acid residues.^{2,3} Previous proteomic study showed a similar pattern,¹ suggesting that this region is prone to degrade. Blo t 5 signal peptide is 17 residues long, as predicted with the SignalP software (Figure S1A), suggesting, together with our mass spectrometry (MS) results, the possibility of a longer signal peptide sequence. To further investigate the importance of this N-terminal motif, recombinant variants of Blo t 5 were produced. The only difference between their sequence was

the presence (rBlo t 5_{long}) or absence (rBlo t 5_{short}) of the N-terminal motif.

Although we have successfully purified completely recombinant soluble nonfusion Blo t 5 variants (Figures S1B,C), some degradation in rBlo t 5_{long} was noticed exactly at the N-terminal region (Figure S2). Even though we added some amino acid residues in this region to increase stability (MGA), and ProtParam tool showed that both variants would be stable, this prediction was not confirmed by our experimental data. Of note, however, the most abundant MS peak (Figure S2) in rBlo t 5_{long} matched with the sequence that was previously used to determine the 3D structure of rBlo t 5.³

Despite both variants have displayed a refolding capacity and similar content of secondary elements (Figure 1B and S1D), the absence of the N-terminal motif improved the folding capacity of Blo t 5_{short}, as our 48h temporal folding analysis showed (Figures 1C and S1E,F). Furthermore, dimerization and/or aggregation of allergens has a direct influence on allergenicity, usually leading to a reduced

FIGURE 1 Physicochemical characterization of nBlo t 5 and recombinant Blo t 5 variants. A: MS identification and superposition of nBlo t 5 peptides after digestion; UniProt and NCBI were the databases used for alignments; B: CD spectra recorded three different conditions; C: CD spectra recorded at 20°C for 48 h. Six out of nineteen-time measurements are shown; D: DLS measurements. R_H : hydrodynamic radius; and E: SDS-PAGE analysis of endolysosomal proteolysis assays of recombinant Blo t 5 variants



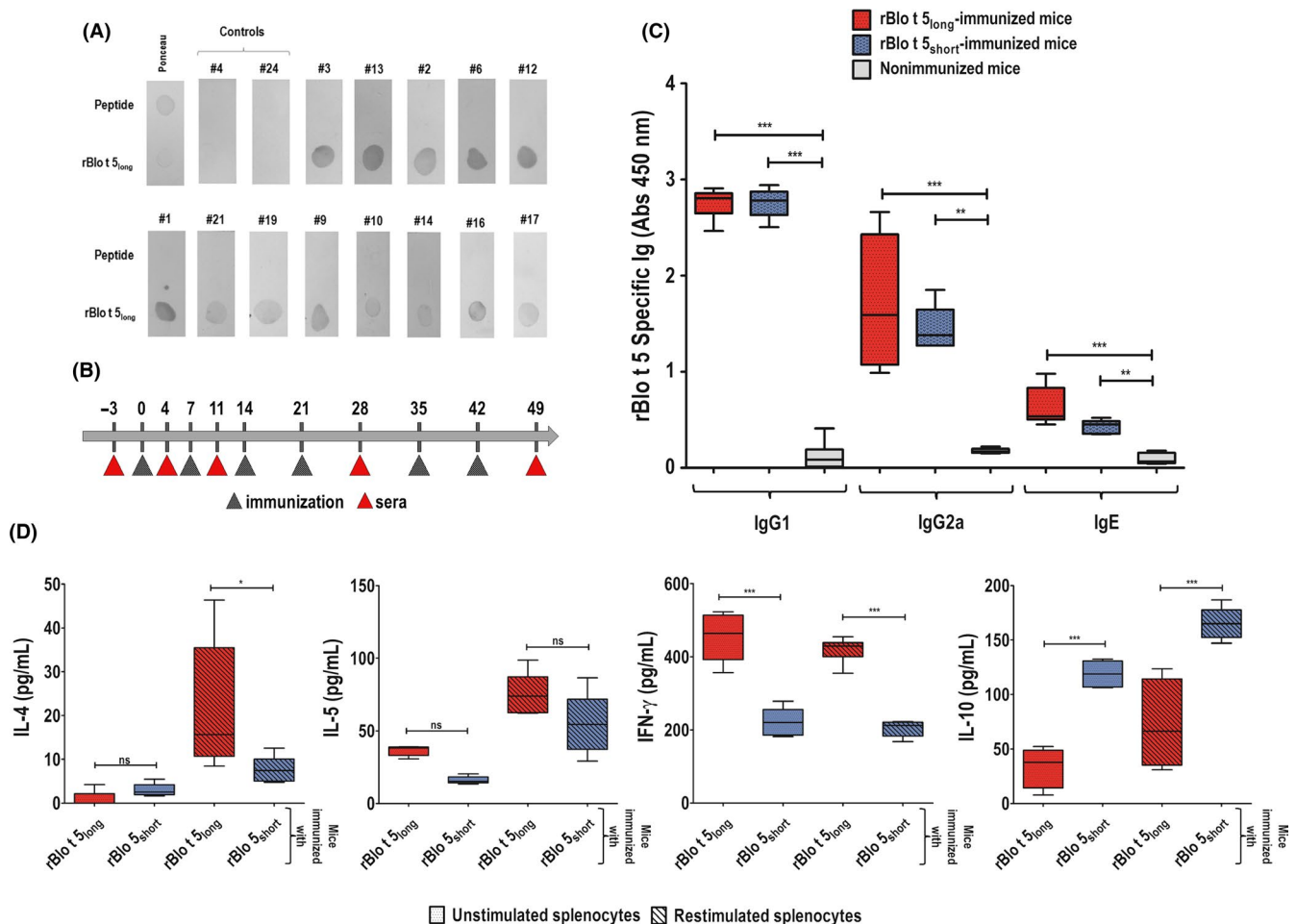


FIGURE 2 Immunological characterization of recombinant Blo t 5 variants. A: Lack of IgE reactivity of dot-blotted Blo t 5-N-terminal peptide (randomly selected sera); For comparison, rBlo t 5_{long} was also dot-blotted. Previously to the addition of a serum, Ponceau staining was performed in each membrane; B: Schematic representation of immunization schedule; C: rBlo t 5 specific IgG1, IgG2a and IgE determined by ELISA using sera from immunized and nonimmunized mice; and D: Measurement of IL-4, IL-5, IL-10 and IFN- γ in splenocytes cultured from immunized animals

IgE binding.⁵ The herein studied rBlo t 5 variants were found to be monomeric in solution (Figure 1D), excluding, therefore, this feature as the responsible for any differences in our in vivo data.

Previous studies demonstrated a decreased susceptibility to endolysosomal proteolysis in proteins with an increased fold stability. This feature may influence allergen processing and, in turn, determine allergenicity through increasing the intensity and polarization of TH2 responses.^{6,7} Our results showed that the shortening of Blo t 5 led to a reduced susceptibility to endolysosomal proteolysis (Figure 1E). We hypothesize that the absence of the disordered N-terminal region in rBlo t 5_{short} has reduced the flexibility of the molecule and, in association with the higher temporal folding capacity, the profile of endolysosomal degradation was then modified. In addition, another study has shown that proteins with an unstructured N-terminus region may be more susceptible to proteolysis. The presence of such region turns cleavage sites more available, facilitating the activity of enzymes.⁸

The identification of the generated proteolytic peptides revealed that the shortening of Blo t 5 did not alter the peptide

cluster formation compared with the full-length molecule (Figure S3). However, the three indicative relevant T-cell epitope regions detected in rBlo t 5_{short} were present for longer time throughout the degradation assay, leading to the assumption that MHC class II loading would be more efficient. The peptide fragments were around the following residues: (a) QHQLDELNENKSKELQEKIIRE; (b) MIEGAQGALERELK; and (c) RFNYEEAQTLSKI. These peptides warrant further investigation to confirm its importance in T-cell proliferation.

We confirmed that rBlo t 5 has no IgE epitopes at the disordered N-terminal motif (Figure 2A), as was reported by other research groups, who located the key amino acids for IgE binding at other positions of the allergen.^{2,3} Moreover, the allergenic properties of Blo t 5 were mostly not changed by shortening the N-terminal region. In fact, the IgE binding in ELISA was not significantly different (Figure S4A). Regarding basophil degranulation, the rBlo t 5 variants showed comparable capacity to induce degranulation (Figure S4B).

Since co-sensitization to *B tropicalis* and *D pteronyssinus* is common in tropical and subtropical countries, we addressed the

IgE cross-reactivity between rBlo t 5_{short} and Der p 5. Prior to our dose-dependent inhibition ELISA, the IgE reactivity of these allergens was evaluated (Figure S4C-E). Cross-inhibition results showed that rBlo t 5_{short} cannot always inhibit the rDer p 5-specific IgE, but rDer p 5 possesses a moderate inhibitory capacity on rBlo t 5_{short}-specific IgE binding (Figure S4F). Previously, it was found only limited cross-reactivity between these allergens. Authors argued that these low levels of cross-inhibition were due to lack of conserved peptide motifs and because they share only 44.3% of amino acid identity.⁹ However, since, in the highest concentrations, rDer p 5 acted as an inhibitor, further studies will be performed by our group, using a higher number of solely sensitized patients, in order to confirm this moderate cross-reactivity.

Although in vitro allergenic assays displayed comparable results, our in vivo evaluation (Figure 2B) strengthens the notion that allergens with low resistance towards endolysosomal degradation may be more allergenic. No statistical differences in antibodies' responses were found between both immunized mice groups; either rBlo t 5_{long} or rBlo t 5_{short} immunized mice showed significantly higher levels of IgG1, IgG2a and IgE in comparison with the negative control group (Figure 2C). Despite no differences in IL-5 levels when mice were immunized with rBlo t 5_{long}, a tendency of higher production was noticed, accompanied by significant higher levels of IL-4 as well as a tendency for more IgE production (Figure 2C,D). Surprisingly, rBlo t 5_{long} induced high levels of IFN- γ (Figure 2D), which make this variant less attractive for AIT because some asthma endotypes are characterized by high levels of this cytokine. In contrast, mice immunized with the proteolytically resistant rBlo t 5_{short} produced significantly more IL-10 and lower IgE levels (Figure 2C,D). The high production of IL-10 is particularly interesting as the cytokine has well-known anti-inflammatory and regulatory functions.

Knowing that lower IgE binding and allergenicity, as well as T-cell priming with reduction of TH2 cytokines and increasing of regulatory cytokines, are features expected in AIT candidate vaccines,⁵ our extensive physicochemical and immunological analyses indicated, therefore, that rBlo t 5_{short} represents a more suitable template for the design of a hypoallergenic derivative. After all, Blo t 5_{short} has shown the following: (a) high solubility; (b) increased folding stability; (c) reduced susceptibility to endolysosomal proteolysis; (d) low IgE induction in vivo; and (e) significantly induces higher production of IL-10.

KEYWORDS

allergens and epitopes, IgE, immunotherapy vaccines and mechanisms

ACKNOWLEDGMENTS

The authors are grateful for the support of the National Council for Scientific and Technological Development of Brazil (CNPq), the Science without Borders Program (grant 200307/2015-0) and to the Coordenação de Pessoal de Nível Superior (CAPES grant 88881.119338/2016-01), as well as the University of Salzburg's Priority Program Allergy-Cancer-BioNano Research Centre. E.

S. Silva was the recipient of a scholarship from the Research's Foundation of the State of Bahia (Fapesb grant BOL0449/2015) and currently is recipient of a postdoc position (Grant n°. 88887.357066/2019-00).

CONFLICT OF INTEREST

E. Silva, M. Alcantara-Neves and C. S. Pinheiro have a patent BR1020180132440 pending. F. Ferreira reports nonfinancial support from Priority Program "Allergy-Cancer-BioNano Research Centre" of the University of Salzburg, during the conduct of the study; personal fees from HAL Allergy, personal fees from Swiss Institute of Allergy and Asthma Research (SIAF), and personal fees from Allergen Online, outside the submitted work. M. Wallner has been supported by the Priority Program of the University of Salzburg and the Austrian National Bank (ÖNB). S. Wuenschmann and M. Chapman are employees of Indoor Biotechnologies Inc and MC is an owner of the company. The remaining authors declare that they have no relevant conflicts of interests.

AUTHOR CONTRIBUTIONS

C. S. Pinheiro, F. Ferreira, N. M. Alcantara-Neves, M. Wallner and G. Gadermaier were involved in conception and design of the study and supervision of the project. E. S. Silva performed the present study, analysed data and conducted the laboratory assays. S. Huber, C. Asam, L. Aglas and J. R. U. Alvarez performed laboratory assays in the Biochemical characterizations. I. Karner performed the CD analysis. P. Briza acquired and analysed the MS data. E. F. Silveira, E. M. M. A. Belitardo and R. T. Torres assisted in the in vivo experiments. S. Wuenschmann and MD Chapman contributed with materials and literature review. E. S. Silva and C. S. Pinheiro drafted the manuscript. All authors revised and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

ONLINE SUPPORTING INFORMATION

N-terminal peptide deletion influences immunological and structural features of Blo t 5

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Materials and methods

Mite culture, protein extract and proteomics analysis

A detailed description of this method was recently published by our group.¹

Expression and purification of Blo t 5 variants

The sequences of the Blo t 5 variants were evaluated by the ProtParam tool (<https://web.expasy.org/protparam/>) and, in order to enhance protein stability and half-life in *E.coli*, two amino acid residues (MGA) were added in the N-terminal region of the hybrid sequence. These sequences were then termed as rBlo t 5_{short} (102 amino acid residues) and rBlo t 5_{long} (119 amino acid residues) and harmonized as previously described.^{2,3} Later on they were synthesized and cloned in a pET 30a+ vector by ATG: biosynthetics (Merzhausen, Germany). The recombinant variants of Blo t 5 were expressed as non-fusion proteins in the *Escherichia coli* strain BL21 StarTM (DE3) (Invitrogen, Carlsbad, CA, USA). Protein expression was performed in supplemented LB medium and the induction of heterologous expression was started by addition of 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) at an OD₆₀₀ of 0.5. After 5h of expression at 32 °C, the cells were harvested at 4000 g, 20 min, 4 °C. The bacterial pellet was resuspended in 50 mM sodium phosphate (NaP) buffer, pH 8 and lysed by four freezing/thawing cycles and homogenized for 5 minutes at highest speed using an Ultra-Turrax T25[®] disperser (IKA, Königswinter, Germany). DNase I (1:1000) was used to degrade the DNA for 2 hours at 4°C. The protein suspension was centrifuged at 13000 g, 20 min, 4 °C and the supernatant was collected. The pellet was resuspended in 6M urea. Evaluation of recombinant expression and solubility was performed using SDS-PAGE (15%).

The proteins were purified by anion exchange chromatography (AEC) using a HiTrap Q FF column (GE Healthcare Biosciences, Little Chalfont, UK) and applying a linear gradient elution (0 to 500 mM NaCl in 50 mM NaP buffer pH 8). The pre-purified samples were further purified by size exclusion chromatography (SEC) in 10 mM NaP buffer, pH 8, using a Superdex GL 75 10/300 column (GE Healthcare Biosciences, Little Chalfont, UK). The purified recombinant variants were cryopreserved until use.

Physicochemical characterization of recombinant Blo t 5 variants

the rBlo t 5 variants were physicochemically analysed, as previously described,⁴⁻⁶ in terms of quantity and identity, amino acid analyses (AAA), mass spectrometry (MS), circular dichroism (CD) and Fourier transform infrared spectroscopy (FTIR).^{5,7,8} CD temporal stability analyses were performed under the same measurement parameters. The proteins were kept at 20°C and measurements were recorded at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, and 48 hours. The variants' aggregation behaviour in solution was revealed by dynamic light scattering (DLS), as previously described.⁵ All the samples used for these analyses were kept in 10 mM NaP buffer, pH 8.

Evaluation of proteolytic stability of rBlo t 5 variants

The susceptibility to endolysosomal proteolysis was assessed by degradome assays.⁹

Donors' sera and related projects

Sera from donors with specific IgE to *B. tropicalis* were selected by positive clinical history, skin prick test and ImmunoCAP (Phadia Diagnostics AB, Uppsala, Sweden). The sera set was obtained from donors enrolled in the following: (i) SCAALA (Social Change in Asthma and Allergy in Latin America) program;¹⁰ ProAR (Bahia State Program for the Control of Asthma and Allergic Rhinitis) program.¹¹ Ethics Committee on Research of the Faculty of Medicine of Federal University of Bahia has approved the present research (CAAE 45376814.0.0000.5577). Moreover, donors from Austria with house dust mite allergy were selected to be part of the cross-inhibition studies. The Ethics Committee of the University of Salzburg (415-E/1398/4-2011) has approved the experiments with patients' sera. In all cases, the donors have signed a written informed consent to be part of this study (Table S2).

IgE reactivity and cross-inhibition analyses

The IgE reactivity of rBlo t 5 variants was verified by ELISA as described previously.⁸ The IgE cut-off was defined according to the mean plus two standard deviations of the results obtained using sera from individuals without history of allergy, class 0 in ImmunoCap and with negative SPT reaction.

In order to verify the IgE reactivity of the N-terminal unstructured region of Blo t 5, the peptide sequence comprising this region (QEHPKPKDDFRNEFDHLLIE) was sent for synthesis to Bachem AG (Bubendorf, Switzerland) and later on analyzed by Dot-blot. A PVDF membrane (Merck Millipore, Billerica, MA, USA) was pre-moistened in 100% methanol for 30 seconds, followed by washing with distilled water (2 min) and tris-buffered saline with 0.05% Tween 20 (TBS-T) for 5 min. An amount of 0.5 µg of either the peptide or rBlo t 5_{long} were applied on the membrane. After drying (1.5h) and Ponceau S staining, the membrane was blocked with 10% BSA. Incubation with the allergic and non-allergic donors' sera (1:10) was performed at 4 °C for 16h. As detection antibody, an alkaline phosphatase-conjugated anti-human IgE antibody (1:10.000) was incubated with the membrane for 2 h at room temperature. Reactive dots were visualized using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrates.

Cross-inhibition assays were performed as described elsewhere.¹² rDer p 5 was obtained in collaboration with Indoor Biotechnologies (Charlottesville, VA, EUA). Only the short variant was used in this evaluation, since the protein was more stable and the N-terminal region, clearly, did not present any IgE reaction in dot blot analysis. It is also important to note that in order to have a better understanding of the IgE cross-inhibition between these two allergens, sera from donors living in a temperate region of the globe were also used in our cross-reactivity studies. In this respect, the IgE reactivity to the allergens in these sera had to be verified prior to the cross-inhibition assays. In this case, 43 and 20 sera from donors living in Brazil and Austria, respectively were used for rDer p 5 IgE reactivity evaluation. Out of this 63, 19 sera were randomly chosen for the cross-inhibition assays.

The reactivity profile of the donors was further confirmed by Western blot. Only the short variant was used in inhibition assay, since the protein was more stable and the N-terminal region, clearly, did not present any IgE reactivity in dot blot analysis.

Mediator release assay

Mediator release assays were performed, as previously described,⁸ using serological IgE antibodies from *Blomia tropicalis* allergic donors and rat basophil leukemia (RBL)-2H3 cells which are transfected with the α chain of the human high-affinity IgE receptor (Fc ϵ RI).¹³

In vivo experiments

BALB/c mice at the age of 8 weeks were used for experiments, as previously published.^{4,8,14-16} Three groups were evaluated: (i) six mice were immunized with PBS+alum; (ii) five mice were immunized with Blo t 5_{short}, and six mice were immunized with Blo t 5_{long}. Sera were taken every two days after each immunization. At day 49, animals were killed, blood and sera were taken. Also, lymphocytes were harvested from the spleen, as previously described.¹⁴ Splenocytes (2×10^5 cells/well) were restimulated with either 40 μ g/mL of either recombinant allergens or pokeweed mitogen (PWN). Secreted cytokines (IL-10, IFN- γ , IL-4 and IL-5) were analysed by OptEIA™ Set ELISA (BD Biosciences, San Jose, USA). Antibody (IgG1, IgG2a and IgE) responses against Blo t 5 were verified by indirect ELISA. All animal experiments were approved by the Ethical Committee for Use of Experimental Animals of the Institute of Health Sciences of Federal University of Bahia (CEUA-ICS 137/2018).

The amount of LPS in recombinant protein samples was quantified by the end-point chromogenic LAL assay (QCL-1000 kit; Lonza, Walkersville, MD, USA). LPS was removed from the sample using Pierce High-Capacity Endotoxin Removal Resin (Thermo Fisher Scientific, Waltham, MA, USA). After removal, the content of LPS was verified again and all samples of the present work were below the limits recommended in the literature.^{17,18}

Statistics

Data normality was determined by the D'Agostino and Pearson test. The distribution was non-normal during human sera related-experiments, then non-parametric tests were used for statistical evaluations. A value of $P < 0.05$ was considered statistically significant. In the case of the mice experiments, the distribution was normal, therefore parametric tests were used to evaluate possible statistical differences.

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Table S1. Molecular properties of *Blomia tropicalis* allergens and their isoforms identified by MS-analysis

Allergen [†]	UniProtKB no.	No. unique peptides	Coverage max	Area (%)	MW (kDa) [‡]	pI [§]	IUIS recognition	Biochemical function/family [¶]
Blo t 1	Q95PJ4	3	14.93	0.72	25.13	8.49	yes	Cysteine protease
	A1KXI0	3	14.41	0.39	36.30	5.54	yes	
Blo t 2	A6XEN8	3	68.31	26.90	13.44	5.91	no	NPC2 Family
	A6XEP1	3	68.31	27.83	13.47	6.20	no	
Blo t 3	A1KXI1	3	6.77	0.09	25.99	7.73	no	Trypsin
Blo t 4	A1KXI2	11	30.83	2.36	55.23	6.52	yes	Alpha amylase
Blo t 5	O96870	6	55.22	2.05	13.89	5.15	yes	Unknown
Blo t 6	A1KXI3	2	9.09	0.11	27.59	5.85	yes	Chymotrypsin
Blo t 8	A1KXG6	7	27.54	0.52	27.50	5.06	no	Glutathione S-transferase
Blo t 10	A7XZI4	22	49.30	14.25	33.03	4.81	yes	Tropomyosin
Blo t 11	Q8MUF6	11	15.77	1.22	101.90	5.65	yes	Paramyosin
	A1KXG8	10	68.46	7.12	14.68	5.64	no	
	A1KXG9	9	51.32	2.57	15.26	5.59	no	
	A1KXH5	6	62.60	3.01	14.73	5.53	no	
Blo t 13	A1KXH5	6	62.60	3.01	14.73	5.53	no	Fatty acid-binding protein
Der p 14-homolog	A7XZI6	11	17.88	0.29	86.41	8.97	no	Apolipoporphin
Der p 15-homolog	A1KXI8	7	22.34	2.03	49.23	5.51	no	Chitinase
Blo t 21	A7IZE9	5	50.39	2.19	13.24	5.35	yes	Unknown
Der p 28-homolog	A1KXJ1	11	24.46	2.50	71.36	5.41	no	Heat shock protein
Tyr p 35-homolog	A1KXI6	12	30.77	3.20	45.11	5.64	no	Aldehyde dehydrogenase
Der p 38-homolog	A1KXI7	2	22.81	0.68	11.54	4.55	no	Unknown

MW, molecular weight; pI: isoelectric point; [†] and [¶] – Allergen's biochemical function was given following these steps: (i) evaluation of the sequence in UniProtKB (<http://www.uniprot.org/>). (ii) isoforms' sequences were checked regarding WHO/IUIS acknowledgment (<http://www.allergen.org>). (iii) in the case IUIS did not recognize the UniprotKB sequence, Allergome platform was used (<http://www.allergome.org>) to assign function/family of the allergen; [‡] and [§] – Only the sequence of the mature chain was used for MW and pI calculations by ProtParam analysis (<https://web.expasy.org/protparam/>). The sequences used for the calculations were selected from UniProtKB, after verification of signal peptide presence using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>).

Table S2. Responsive characteristics of donors included in the present study

Donor number [†]	SPT [‡]		ImmunoCap <i>Blomia tropicalis</i> [§]		ImmunoCap <i>Dermatophagoides pteronyssinus</i> [¶]		IgE reactivity ^{††}	
	<i>Blomia tropicalis</i>	<i>Dermatophagoides pteronyssinus</i>	kU/L	Class	kU/L	Class	rBlo t 5 _{short}	rDer p 5
#1	NA	NA	> 100	6	> 100	6	+	ND
#2	+	+	68.3	5	NA	NA	+	+
#3	+	+	50.9	5	NA	NA	+	+
#4	+	+	59.2	5	NA	NA	-	+
#5	+	+	62.1	5	NA	NA	-	+
#6	+	+	30.9	4	NA	NA	+	ND
#7	+	+	40.1	4	12.4	3	+	+
#8	+	+	32.8	4	10.4	3	+	+
#9	+	+	18	4	5.27	3	+	+
#10	+	+	31.7	4	7.3	3	+	+
#11	+	+	20	4	3.08	2	+	+
#12	NA	NA	17.6	4	0.74	2	+	+
#13	+	+	41.9	4	27	4	+	+
#14	+	+	9.2	3	NA	NA	+	+
#15	+	+	11.2	3	NA	NA	+	+
#16	+	+	8.51	3	NA	NA	+	+
#17	+	+	12.8	3	6.18	3	+	+
#18	-	+	1.66	2	32.3	4	-	+
#19	+	+	1.25	2	NA	NA	+	+
#20	+	+	3.42	2	NA	NA	-	+
#21	+	+	2.19	2	26.5	4	+	+
#22	-	-	0.1	0	NA	NA	-	-

Table S2. Continued

Donor number [†]	SPT [‡]		ImmunoCap <i>Blomia tropicalis</i> [§]		ImmunoCap <i>Dermatophagoides pteronyssinus</i> [¶]		IgE reactivity ⁺⁺	
	<i>Blomia tropicalis</i>	<i>Dermatophagoides pteronyssinus</i>	kU/L	Class	kU/L	Class	rBlo t 5 _{short}	rDer p 5
#23	-	-	0.1	0	-	-	-	-
#24	-	-	0.1	0	-	-	-	-
#25	-	-	0.1	0	0.29	0	-	-
#26	+	+	3.97	2	8.49	3	-	+
#27	+	+	9.29	3	17.8	4	-	+
#28	NA	NA	1.34	2	2.52	2	+	+
#29	NA	NA	8.52	3	4.89	3	+	+
#30	NA	NA	4.11	3	0.1	0	+	-
#31	NA	NA	49.6	4	1.8	2	+	-
#32	NA	NA	> 100	6	> 100	6	+	+
#33	NA	NA	15.3	3	15.8	3	+	+
#34	NA	NA	73.9	5	6.85	3	+	+
#35	NA	NA	2.37	2	0.59	1	+	+
#36	NA	NA	26.5	4	7.8	3	+	+
#37	NA	NA	0.95	2	1.04	2	+	-
#38	NA	NA	22.1	4	7.63	3	+	+
#39	NA	NA	33.2	4	9.93	3	+	+
#40	NA	NA	2.03	2	5.92	3	+	+
#41	NA	NA	1.59	2	0.3	0	+	+
#42	NA	NA	> 100	6	> 100	6	+	+
#43	NA	NA	61.9	5	3.06	2	+	+
#44	NA	NA	4.64	3	22.1	4	+	+

Table S2. Continued

Donor number [†]	SPT [‡]		ImmunoCap <i>Blomia tropicalis</i> [§]		ImmunoCap <i>Dermatophagoides pteronyssinus</i> [¶]		IgE reactivity ⁺⁺	
	<i>Blomia tropicalis</i>	<i>Dermatophagoides pteronyssinus</i>	kU/L	Class	kU/L	Class	rBlo t 5 _{short}	rDer p 5
#45	-	-	0.1	0	0.1	0	-	-
#46	-	-	0.1	0	0.1	0	-	-
#47	-	-	0.1	0	0.1	0	-	-
#48	-	-	0.1	0	0.1	0	-	-
#49	-	-	0.2	0	0.2	0	-	-
#50	NA	NA	95.8	5	1.67	2	+	+
#51	NA	NA	61.4	5	33.6	4	+	+
#52	NA	NA	8.4	3	2.47	2	+	+
#53	NA	NA	28.8	4	58.6	5	+	+
#54	NA	NA	56.5	5	84.2	5	+	-
#A1	NA	+	NA	NA	>100	6	+	+
#A2	NA	+	NA	NA	>100	6	+	+
#A3	NA	+	NA	NA	>100	6	+	+
#A4	NA	+	NA	NA	>100	6	+	+
#A5	NA	+	NA	NA	>100	6	+	+
#A6	NA	+	NA	NA	>100	6	+	+
#A7	NA	+	NA	NA	>100	6	+	+
#A8	NA	+	NA	NA	>100	6	-	+
#A9	NA	+	NA	NA	>100	6	+	+
#A10	NA	+	NA	NA	>100	6	-	-

Table S2. Continued

Donor number [†]	SPT [‡]		ImmunoCap <i>Blomia tropicalis</i> [§]		ImmunoCap <i>Dermatophagoides pteronyssinus</i> [¶]		IgE reactivity ^{††}	
	<i>Blomia tropicalis</i>	<i>Dermatophagoides pteronyssinus</i>	kU/L	Class	kU/L	Class	rBlo t 5 _{short}	rDer p 5
#A11	NA	+	NA	NA	>100	6	+	+
#A12	NA	+	NA	NA	>100	6	+	+
#A13	NA	+	NA	NA	>100	6	+	+
#A14	NA	+	NA	NA	>100	6	+	-
#A15	NA	+	NA	NA	>100	6	-	-
#A16	NA	+	NA	NA	>100	6	+	-
#A17	NA	+	NA	NA	>100	6	-	-
#A18	NA	+	NA	NA	>100	6	+	+
#A19	NA	+	NA	NA	>100	6	+	+
#A20	NA	+	NA	NA	>100	6	+	+

In bold – healthy donors used as controls;

† – The letter A in the donor's number refers to the fact that the serum was obtained from a person living in Austria. All the other sera were collected in Brazil;

‡ – SPT: skin prick test. The test was considered to be positive (+) if the diameter of the wheal was at least 3 mm greater than the saline control;

§ – IgE specific-levels were measured by ImmunoCAP (D201, Phadia Diagnostics AB, Uppsala, Sweden);

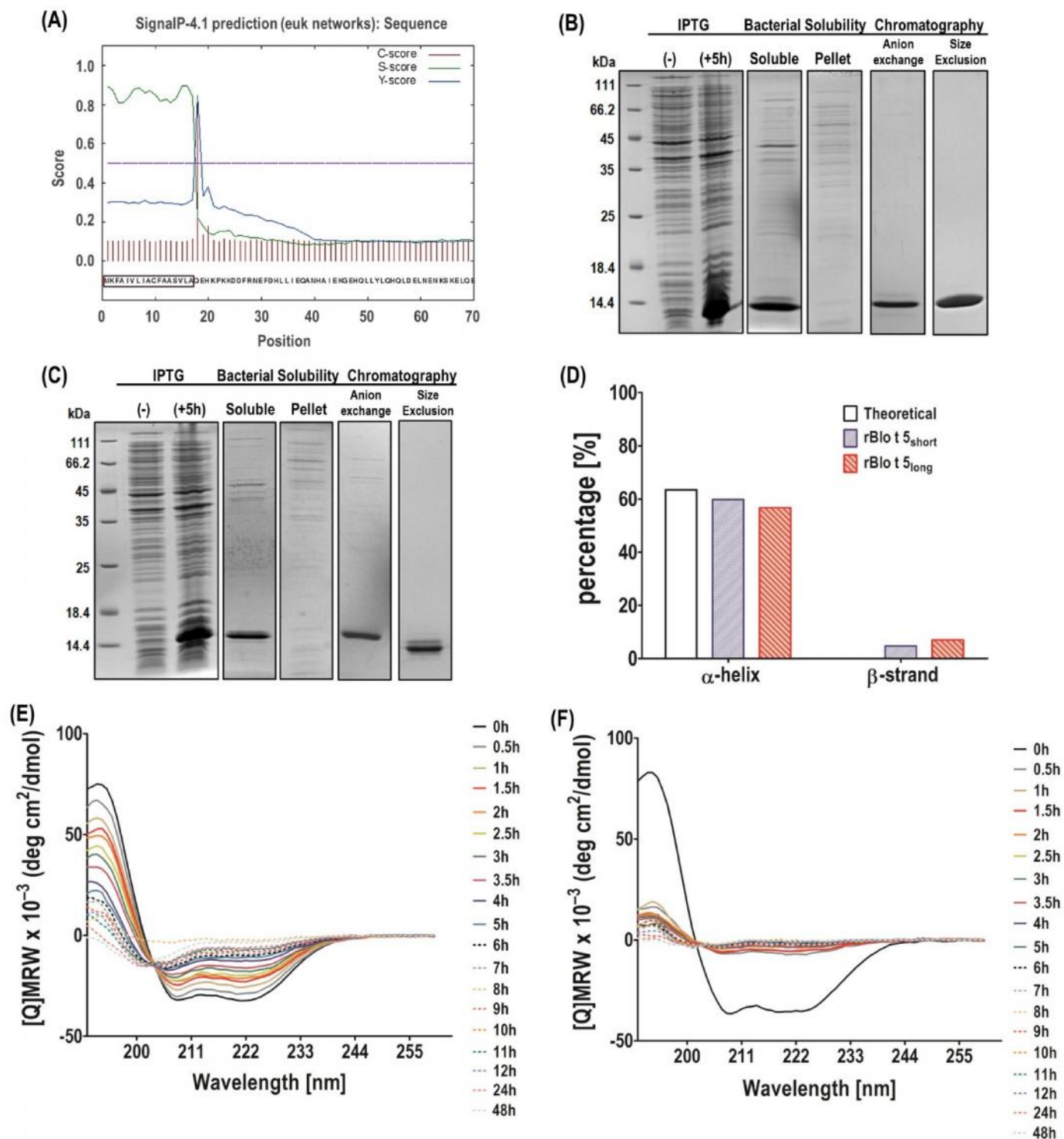
¶ – IgE specific-levels were measured by ImmunoCAP (D1, Phadia Diagnostics AB, Uppsala, Sweden);

†† – The reaction was considered to be positive (+) if the relative light unity (RLU) was higher than the cut-off. rBlo t 5_{short} cut-off: 9,56E+03. rDer p 5 cut-off: 1,50E+04. The cut-off was determined as the mean plus two standard deviations of the results obtained using sera from healthy donors;

NA – not available;

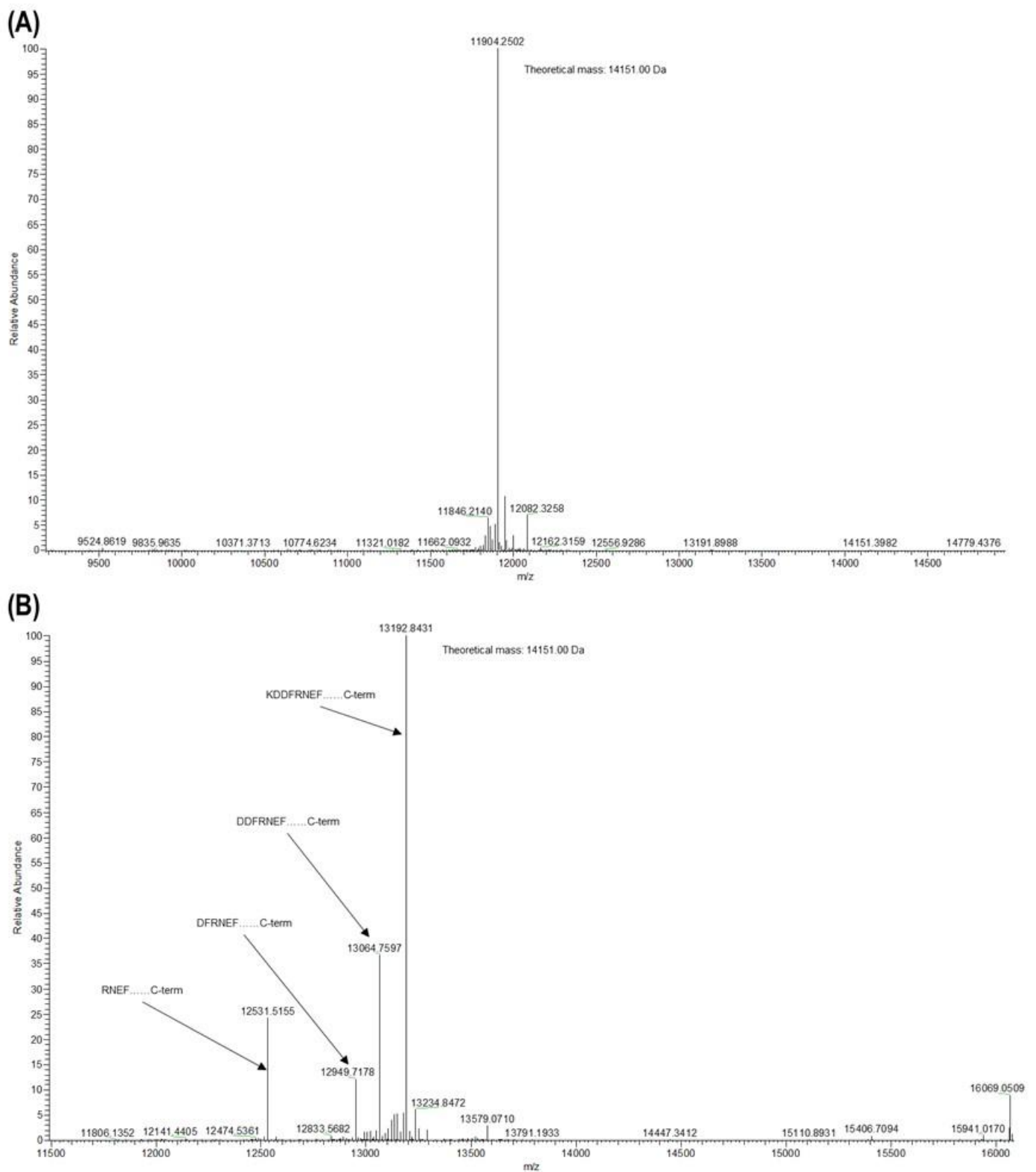
ND – not determined because there was no sera left for the IgE reactivity and inhibition assays;

Figure S1



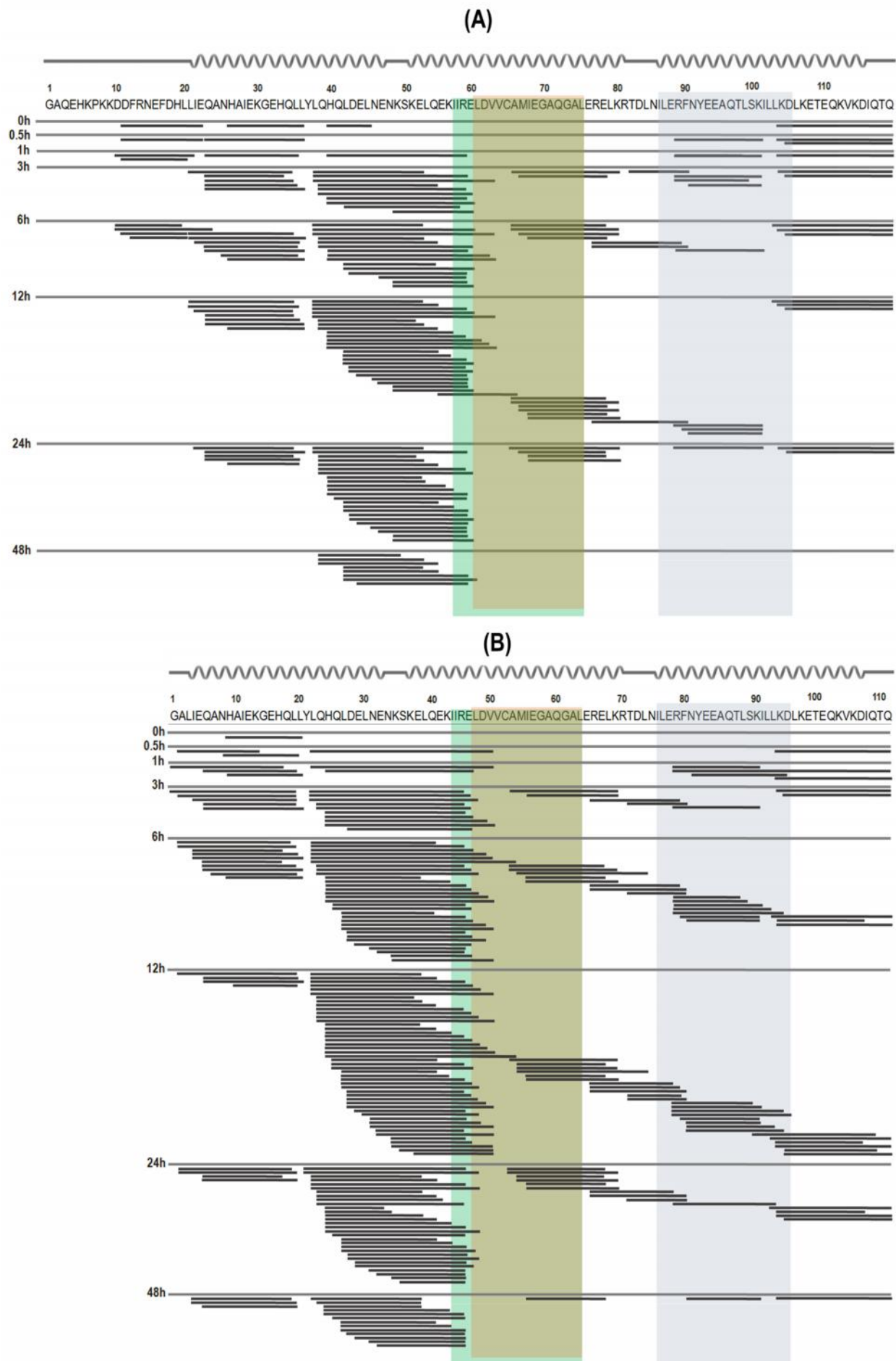
Structural characterization of recombinant Blo t 5 variants. **A:** Signal peptide prediction using SignalP 4.1 server; **B and C:** Heterologous expression and purification of rBlo t 5_{short} and rBlo t 5_{long} respectively; **D:** FTIR spectra were used to calculate the secondary structure elements displayed here; the theoretical values were based on the sequence available in Uniprot, ID: O96870; **E and F:** Temporal folding CD-analyses of rBlo t 5_{short} and rBlo t 5_{long}, respectively recorded at 20 °C for 48h.

Figure S2



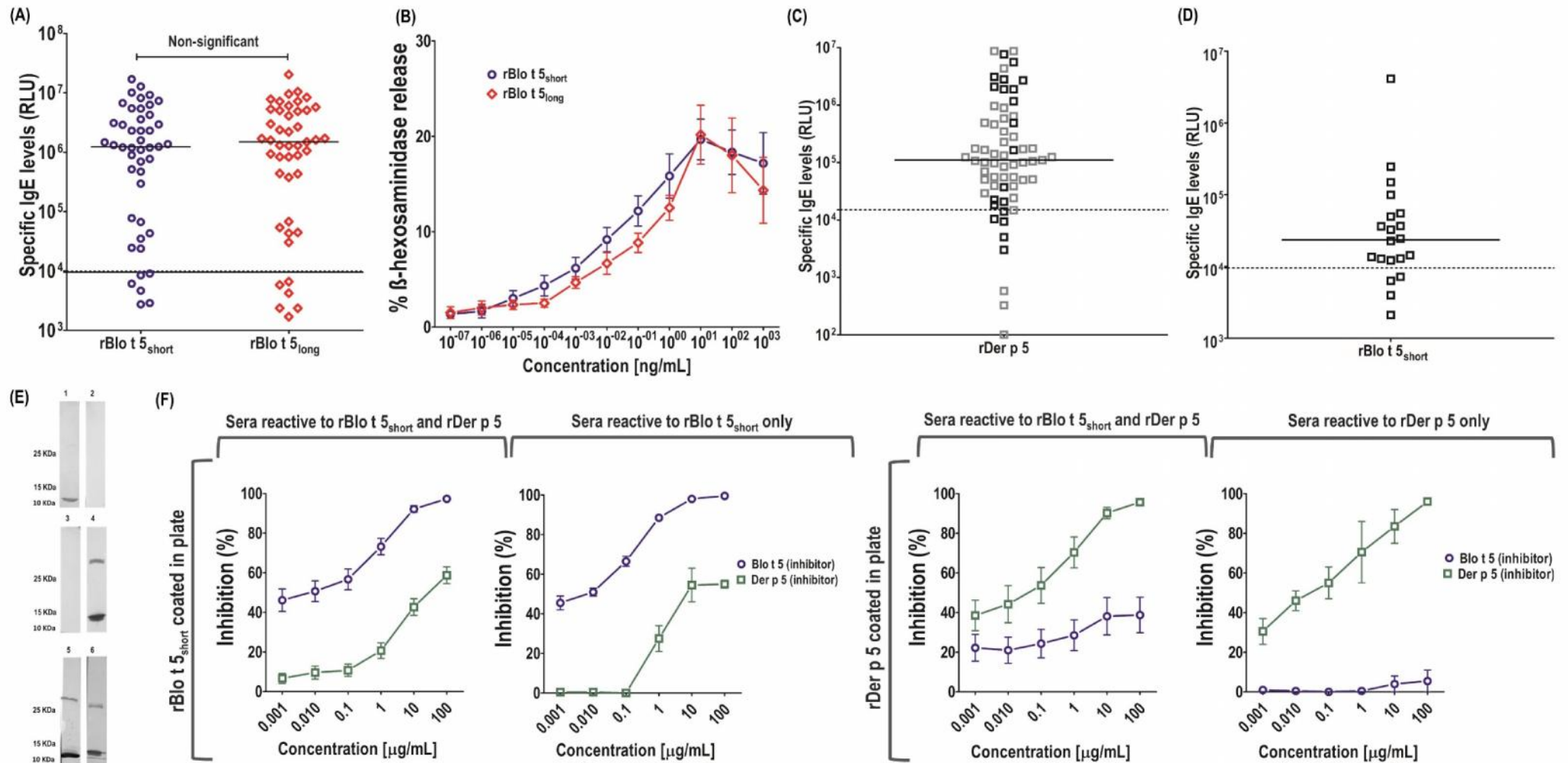
Mass spectrometry analysis of the purified rBlo t 5_{short} (A) and rBlo t 5_{long} (B). Arrows indicate the degradation of Blo t 5_{long}. After tryptic digestion, the amino acid sequence of each peak was identified as it is shown.

Figure S3



Blo t 5 variants peptide cluster pattern derived from in vitro endolysosomal degradation. Peptides sequenced by tandem mass spectrometry after 48 h of in vitro digestion are shown for rBlo t 5_{long} **(A)** and rBlo t 5_{short} **(B)**. Coloured bars indicate T-cell epitopes of Blo t 5, previously published.^{19,20} Note that the two first are overlapping because the two peptides sequences were slightly different in the two studies.^{19,20} Positions of alpha-helical motifs are shown on the top.

Figure S4



IgE responses to group 5 mite allergens. **A:** Specific IgE reactivity determined by ELISA in *B. tropicalis* allergic donors' sera ($n=45$); RLU: relative light units; Dashed line represents Blo t 5_{long} cut-off (1,07E+04 RLU); Solid line represents Blo t 5_{short} cut-off (9,58E+03 RLU). **B:** Titration curve of six mediator release assays using huBL cells and *B. tropicalis* allergic donors' sera. Specific IgE reactivity for rBlo t 5_{short} (**C**) and rDer p 5 (**D**) determined by ELISA; Symbols in grey and black colours represent sera from donors living in Brazil ($n=43$) and Austria ($n=20$), respectively; rBlo t 5_{short} cut-off: 9,56E+03. rDer p 5 cut-off: 1,50E+04; **E:** Examples of solely and doubly reactive sera were confirmed by Western blot analyses; Lanes 1, 3 and 5 show the IgE reaction to rBlo t 5_{short}, while lanes 2, 4 and 6 show the IgE reaction to rDer p 5; A pool of two sera solely sensitized to rBlo t 5_{short} was used in the lanes 1 and 2, whereas the two serum donors that reacted only to rDer p 5 were used in the lanes 3 and 4; In the lanes 5 and 6, the double positive sera (randomly selected) were used in the assay. **F:** A cross-inhibition ELISA with rBlo t 5_{short} in the solid phase and rDer p 5 in the solid phase; Concentration-dependent IgE inhibition curves are shown on for double sensitized sera ($n=15$, randomly chosen), as well as solely sensitized to rBlo t 5 or rDer p 5 (both $n=2$); For all assays, a dose-dependent IgE inhibition was performed after serum pre-incubation with rBlo t 5_{short} and rDer p 5;